RESEARCH ARTICLE



# Between approval and disapproval: Citizens' views on the invasive tree Ailanthus altissima and its management

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#### Abstract

While cities are invasion hotspots, the view of urban residents on non-native species is critically understudied – an important knowledge gap since strategies on biological invasions could gain power by integrating human values, attitudes and perceptions. How citizens perceive the non-native tree *Ailanthus altissima* (tree of heaven) is unknown despite its abundance in many cities globally and its classification as invasive in many countries. In a quantitative survey with closed questions, we analysed (i) whether residents of Berlin, Germany knew the widespread species, (ii) how they perceived it in different urban situations, (iii) how they accepted different management strategies of it, and (iv) how the sociodemographic background of respondents predicted their preference and acceptability ratings.

In total, we surveyed 196 respondents. Most respondents recognized the tree in a photograph, but few provided its correct name. Citizens' preferences differed significantly among four urban contexts in which the species was shown, with prevailing approval for trees as a component of designed green spaces and less pronounced preferences for wild-grown trees in other urban spaces. When respondents were asked to indicate how the tree should be managed (three options), we found the most support for removal in problematic cases ('adaptive on-site' strategy); some support was found for the 'leave alone' strategy and least support for the 'complete removal' management strategy. Practitioners with expertise in urban landscaping were more critical of *Ailanthus* than laypeople. Ordinal logistic regression analyses showed that respondents with a 'close to nature' behaviour and attitude had a more positive view on *Ailanthus* and expressed more support for 'leave alone' management. Results demonstrate the importance of citizens' context dependent views about a widespread invasive species, spanning from approval to disapproval in

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different situations. We conclude that urban management strategies concerning *Ailanthus* would gain support from citizens when combining multiple approaches: (i) to control the species in case of realized negative impacts; (ii) to prevent the invasion of the species in areas of conservation concern; and (iii) to develop novel approaches of integrating wild *Ailanthus* trees into urban green spaces. These insights could support management measures that need to be established due to the EU-Regulation on Invasive Alien Species.

#### **Keywords**

acceptance, biodiversity valuation, invasive alien plant species, management strategies, public perception, urban green spaces, weed control, wild vegetation, xenophobia

# Introduction

Biological invasions fundamentally have a human dimension because non-native species are defined as those species that humans have introduced into areas beyond their natural range (Essl et al. 2018). Many invasion studies therefore illuminate the role of humans as the driving force of invasions and analyse, for example, the pathways of introduction, transport and release of non-native species (Thellung 1915; Kowarik 2003; Hulme 2009; Kueffer 2017). Another important facet of the intersection between humans and biological invasions, however, is clearly understudied: people's view on non-native species and related management policies (Sharp et al. 2011; Abrahams et al. 2019; Kapitza et al. 2019).

Strategies on the management of biological invasions, ranking high on local, national and international agendas (Essl et al. 2020; Pyšek et al. 2020), could gain power through increased integration of human values, attitudes and perceptions (Crowley et al. 2017; Shackleton et al. 2019a), as generally posited for conservation strategies (Manfredo et al. 2017). Considering the socio-ecological context in the complex scenarios of species' invasions and their potential management could help transfer approaches from broader to more local scales and vice versa, and enhance their acceptance and efficiency (Crowley et al. 2017; Woodford et al. 2018; Shackleton et al. 2019b). This is important because whether and how introduced species are managed is highly debated and often evokes disapproval in society, especially when it comes to charismatic species (Fischer and Young 2007; Selge et al. 2011; Verbrugge et al. 2013; Novoa et al. 2017; Höbart et al. 2020; Jarić et al. 2020).

Views on non-native species and their management starkly differ among and within groups of the public, scientists and different stakeholders (e.g., Fischer et al. 2014; Lindemann-Matthies 2016; Novoa et al. 2016; Heink et al. 2018; Luna et al. 2019; Cordeiro et al. 2020; Gbedomon et al. 2020). Thus, transparency of values, beliefs and attitudes that underlie the assessment of non-native species is required to make impact assessments and related strategies traceable (Estévez et al. 2015; Bartz and Kowarik 2019).

Preference studies on the species level are generally still scarce in the urban context (Botzat et al. 2016). Yet a small but increasing number of studies have explored the public's view, or that of different stakeholders, on non-native species, their impacts and

related management strategies, as recently reviewed by Kapitza et al. (2019) and Shackleton et al. (2019b). Most previous studies have been conducted in rural environments or on a country scale (e.g., Kowarik and Schepker 1998; Andreu et al. 2009; Selge et al. 2011; Sharp et al. 2011; Fischer et al. 2014; Crête et al. 2020), while urban studies are scarce (but see Verbrugge et al. 2013; Lindemann-Matthies 2016; Shackleton and Shackleton 2016; Potgieter et al. 2019a; Nguyen et al. 2020; Shackleton and Mograbi 2020). This leads to a surprising paradox: the human perspective on biological invasions is least understood for cities – places where both the human population and the number of introduced species reach their peak (Pyšek 1998; Kühn et al. 2004). Cities are introduction hubs of non-native species and can be invasion foci for adjacent landscapes, and non-native species can conflict with biodiversity conservation or ecosystem provisioning in cities (Gaertner et al. 2017; Shackleton et al. 2019b; Kowarik and Fischer 2021).

Non-native species can, however, also play a beneficial role in cities by supporting a wealth of urban ecosystem services (Dickie et al. 2014; Potgieter et al. 2017; Vaz et al. 2018; Shackleton et al. 2019b; Schlaepfer et al. 2020). Cultivated non-native plants are an important component of urban green infrastructure (Petřík et al. 2019; Schlaepfer et al. 2020), and sometimes grow under harsh urban conditions where there are few alternatives among native species (Sjöman et al. 2016). Planting and maintaining non-native species in parks and gardens directly reflect how people appreciate them for aesthetical and other reasons (Lindemann-Matthies 2016; Vaz et al. 2018). In parallel, non-native species contribute to regulating and cultural ecosystem services to the same extent as native species, as Schlaepfer et al. (2020) quantified for Geneva, Switzerland. Comprehensive assessments of non-native species in urban settings thus need to consider the benefits of these species for urban societies alongside the trade-offs with conservation risks or other disservices (van Wilgen 2012; Dickie et al. 2014; Potgieter et al. 2019b; Shackleton et al. 2020). This necessitates a combination of approaches from ecology and the social sciences (Crowley et al. 2017; Shackleton et al. 2019c).

As a contribution to such approaches, we analysed the view of citizens on the non-native tree Ailanthus altissima (tree of heaven, henceforth Ailanthus) in Berlin, Germany. Our model species has been classified as invasive in many countries because it can threaten biodiversity, for example, in dry grassland or rocky habitats (e.g. Pergl et al. 2016). Ailanthus has been designated by experts as one of "100 of the World's Worst Invasive Alien Species" (ISSG 2017), but is not among the worst invasive species in Europe according to the results of an impact scoring approach (Nentwig et al. 2018). Nevertheless, in 2019 the species has been included in the "List of Invasive Alien Species of Union Concern" (European Commission 2019). It thus falls under the "Regulation (EU) No 1143/2014 on the prevention and management of the introduction and spread of invasive alien species [IAS]" (European Parliament 2014). As for all listed widespread invasive species at present, each member state of the European Union must develop and implement management measures for Ailanthus, which should be "appropriate to the specific circumstances" and "based on an analysis of costs and benefits" (European Commission 2019). The development and implementation of such measures not only require ecological knowledge, but also the inclusion of people's views on

the respective species and possible management strategies (Brundu 2017). Therefore, our study aimed at understanding the views of citizens on *Ailanthus* in Berlin.

Since its introduction from China to Europe around 1750, *Ailanthus* has been planted in many cities (Kowarik and Säumel 2007). These plantings were the source for often prolific wild (i.e., spontaneous) populations in cities and along transportation corridors outside cities (Kowarik and Säumel 2007; McAvoy et al. 2012; Casella and Vurro 2013; Kim 2016; Walker et al. 2017; Luigi Nimis et al. 2019; Paź-Dyderska et al. 2020). Some rural populations also exist in forest gaps (e.g. Knüsel et al. 2019; Lapin et al. 2019; Wagner et al. 2020), but these are usually less abundant in central Europe than urban populations. While there is little evidence of negative effects of *Ailanthus* on urban biodiversity, wild urban populations can be challenging due to the vigorous clonal growth and regeneration of the tree, necessitating increased efforts for maintaining green spaces, transportation corridors and built structures (Kowarik and Säumel 2007; Sladonia et al. 2017), including ancient monuments in southern Europe (Celesti Grapow and Ricotta 2020; Trotta et al. 2020).

The services and disservices that people gain from nature usually relate to societal values (Scholte et al. 2015). However, urban residents' views on non-native species such as *Ailanthus* in urban environments is largely unexplored. The few existing studies on citizens' views on non-native plants mostly refer to a species' identity, i.e. they explore respondents' preferences for a species as is (e.g. Verbrugge et al. 2013; Lindemann-Matthies 2016; Shackleton and Shackleton 2016; Potgieter et al. 2019a; Shackleton and Mograbi 2020), without differentiating for the spatial or functional context of a species within a city. Yet context matters when it comes to the specific invasion impacts and disservices of non-native species (Pyšek et al. 2012; Kumschick et al. 2015; Shackleton et al. 2019c). This leads to the challenge of differentiating impact assessments of non-native species for a range of environmental, spatial and functional settings (Bartz and Kowarik 2019).

We hypothesised that context dependence also matters for how urban residents view widespread invasive species, leading to different preferences of *Ailanthus* trees in different urban settings. In a quantitative survey with closed questions and photographic stimuli presenting the tree in different urban contexts, we assessed people's views on *Ailanthus* in relation to (a) the urban setting, (b) potential management strategies and (c) urban resident's sociodemographic backgrounds (i.e., gender, age, professional context; Fig. 1, Table 1).

Urban authorities are often confronted with the question of whether and how to manage *Ailanthus*. This question becomes even more important because management measures need to be implemented in the European Union according to the EU-legislation on invasive species (Brundu 2017). Hence, we asked for the acceptability of three management options (differing in their severity) of *Ailanthus* in the urban environment (Fig. 1, right below). Previous studies have shown that values attributed to nonnative species, or support for different management strategies, can depend on a range of sociodemographic variables such as age and gender, the level of knowledge in the field of study, and respondents' nature relatedness (Garcia-Llorente et al. 2011; Selge



**Figure 1.** Approach of the study aiming to understand (i) respondents' preferences of *Ailanthus altissima* in different urban contexts (1–4, right on top); (ii) respondents' acceptability of different management strategies for *Ailanthus* (1–3, right, below), (iii) interactions between preference and acceptability (indicated by the two arrows), and (iv) characteristics of the respondents as predictors for preferences and acceptability (left part of the figure).

et al. 2011; Sharp et al. 2011; Lindemann-Matthies 2016; Shackleton and Shackleton 2016; Potgieter et al. 2019a). We thus included a set of sociodemographic variables and variables that assessed respondents' 'close to nature' behaviour and attitude as potential predictors of respondents' views on *Ailanthus* (Fig. 1, left). Since environmental preferences can differ between laypeople and those with professional expertise in the field (Bardsley and Edwards-Jones 2006; Hofmann et al. 2012; Gifford and Nilsson 2014; Shackleton et al. 2019a, b), we differentiated between two groups in our survey: respondents that had been randomly approached in Berlin's open spaces (henceforth laypeople) and vocational students with professional experience in urban landscaping, including green space management (henceforth practitioners).

In summary, we addressed the following research questions: (1) Do urban residents recognise *Ailanthus* and can they provide its name? (2) How do urban residents prefer *Ailanthus* in four urban contexts, which depict it specifically as either a cultivated or a wild-growing tree? (3) How do urban residents accept three strategies about managing *Ailanthus*, i.e. 'leave alone', 'adaptive on-site' or 'complete removal' management? (4) How do respondents' (i) knowledge (self-estimated, assessed and provided) of *Ailanthus*, (ii) 'close to nature' behaviour and attitude, and (iii) sociodemographic background (including their practitioner vs. layperson status) predict their preference of *Ailanthus* in different contexts (question 2) and their acceptance of management strategies (question 3)?

**Table 1.** Full list of predictor variables used to assess the context-dependent preferences and acceptability of management strategies for *Ailanthus*. The original wording and questions are given in a Suppl. material 1. For an overview on how the respondents' age and gender (g, h) are distributed in relation to professional context (i), see Table 2. For how the predictor variables relate to the response variables, see results in Tables 3, 4.

Predictor variable	Question/Explanations	Variable type
a) Self-estimated knowledge	Do you know this tree?	Binary:
	(shown on a photograph)	0 = no
		1 = yes
		(NA = do not know)
b) Assessed knowledge	Do you know the name of the tree?	Binary:
	(from the photograph)	0 = no (person did not provide the
		correct colloquial or Latin name)
		1 = yes (person provided the correct
		colloquial or Latin name)
		(NA = do not know)
c) Provided knowledge	Half of the respondents received the additional information:	Binary:
	"This non-native tree of Chinese origin". This was only	0 = no
	analysed for the acceptability of management strategies.	1 = yes
d) Gardening	Respondents gardening activity (e.g. in a garden, on the	Binary:
	balcony, in a community garden)	0 = no
		1 = yes
e) Visit of urban green area	Frequency of visit of a public urban green area (e.g. park,	Categorical:
-	forest, playground, cemetery, waterfront, etc.)	0 = never
		1 = less than once a week
		2 = once a week
		3 = several times a week
		NA = do not know
f) Role of nature	Role of nature when visiting a public urban green area	Categorical:
		0 = not
		1 = a little
		2 = moderately
		3 = quite a bit
		4 = very
g) Age	Respondents' age	Categorical:
		1 = younger than 30 years
		2 = between 30 and 60 years
		3 = older than 60 years
h) Gender	Respondents' gender	Categorical:
	1 0	1 = male
		2 = female
		3 = diverse
i) Professional context	Whether respondents were practitioners or randomly	Binary:
	approached citizens which were passing-by in a green space	1 = practitioners
	or public square	2 = laypeople

# Methods

# Study region

Berlin is Germany's capital and largest city, with 3.7 million inhabitants within a total area of 891.1 km<sup>2</sup>. The climate is temperate, with an annual average temperature of 9.9 °C and a mean annual precipitation of 576 mm, with increasing periods of heat and drought in the observation period of 1981–2010 (Cubasch and Kadow 2011).

Berlin represents a metropolitan region, as today's Berlin is a result of the unification of several cities and other settlements in 1920. The resulting polycentric urban structure includes manifold remnants of the natural landscape and the preindustrial cultural landscape, which are located between individual settlement cores and at their peripheries. In addition to designed green spaces within the built areas, a new type of urban nature emerged from the natural revegetation of vacant land after the Second World War (WW II) and has been partly integrated into Berlin's green infrastructure (von der Lippe et al. 2020). About 59% of Berlin's surface is dominated by built-up areas and streets, while green and blue spaces cover 41% of the area (SDUDH 2016).

# Model species

Ailanthus has been cultivated as an ornamental species in Berlin since the 1780s. Today, Berlin's tree database reports 3,004 Ailanthus trees along streets and in public green spaces (SDUDH 2020). In addition, there is a large, but unknown quantity of cultivated and wild trees in the same or other land-uses types. Ailanthus is a dioecious species, with female trees producing large quantities of seeds that are spread by wind as the primary dispersal vector (Kowarik and Säumel 2008; Wickert et al. 2017). In the post-war period since 1945, cultivated female trees serving as propagule sources, in combination with the high availability of open sites, facilitated the onset of invasion processes in Berlin and other cities subjected to destruction during WW II (Kowarik and Böcker 1984). At the beginning of the 1980s, Ailanthus was already a common wild tree in Berlin, mostly in built-up areas, in green spaces and along urban transportation corridors (Kowarik and Böcker 1984). Today, the populations have become more prolific in many parts of the city (Seitz et al. 2012), but are largely absent in near-natural ecosystems (Kowarik et al. 2013). In some urban sites, successional processes resulted in a novel forest type dominated by *Ailanthus*, but much less frequently than emerging forests with other dominant species (Kowarik et al. 2019). Wild populations in green spaces are often abundant due to adjacent seed sources and clonal offspring. The colonization of urban transportation corridors (road verges, tree pits, rail lines) is facilitated by secondary wind dispersal (Kowarik and von der Lippe 2011) and traffic (von der Lippe et al. 2013). Despite the abundance of Ailanthus in Berlin, no conflicts with biodiversity conservation have been reported thus far (unpublished data). Nevertheless, many spontaneous populations are managed in open urban spaces, likely due to conflicts with aesthetical values or for practical reasons, e.g. when blocking the view at roadsides.

# Study design and field survey

In line with our research questions, we developed a quantitative study approach that combined theory from ecology and the social sciences (i.e. knowledge, context, sociodemographic data; Shackleton et al. 2019c). We devised a questionnaire with embedded photographic stimuli of *Ailanthus* in different urban situations. The questionnaire was pre-tested prior to the field survey with  $N_{pre} = 10$ , which led to a few adjustments

in the phrasing of the sociodemographic questions to improve the general understanding. There were no institutional requirements for ethical clearance and the survey was undertaken in accordance with the General Data Protection Regulation (GDPR) of the European Union.

In the field survey in 2019 (i.e., before the COVID-19 pandemic), we included both laypeople and practitioners in the field of urban gardening and landscaping. As practitioners, we approached students of the "Peter-Lenné-Schule", the Berlin vocational school for training in the field of urban gardening and landscaping. This is usually a type of secondary school with students switching regularly between their practical education partner (often a private company), where they gain hands-on experience, and the school, where they learn the theoretical background for their field of expertise. This type of school also offers courses to professionals in extraoccupational training programs wishing to further their expertise or earn a professional degree. During summer 2019 we first interviewed 96 students who were being trained in the field of urban landscape gardening. Of these, 14% had already completed vocational training before starting school. About two thirds of the surveyed students (63%) said that they had already worked in the public sector of landscape gardening, which strongly relates to green space management. Due to their practical work experience and specific educational background, we assumed that this group had more experience with management challenges regarding Ailanthus than the group of randomly interviewed people that we approached as laypeople. The survey was undertaken with the students in the classroom either at the beginning or end of their lesson. The questionnaires were handed out to the students with the request not to communicate among themselves. The time limit for answering the questionnaire was ten minutes.

Second, we performed standardized, structured interviews with randomly approached people in public spaces that we expected to be laypeople. Each interview lasted between five and ten minutes and included the same survey instrument and stimuli used in the practitioner group. To achieve a broad distribution in the Berlin population, several places in Berlin and different times of the day (from early mornings to evenings) and days of the week (both weekdays and weekends) were selected for the surveys of passers-by. The selected places included urban green spaces (57.4%) and public spaces and city plazas (42.6%) in different districts of Berlin (Alice-Salomon-Platz, Kienbergpark, Hildegard-Knef-Platz, Mariannenplatz, Tempelhofer Feld, Rüdesheimer Platz, Wittenbergplatz, Treptower Park). Areas with high tourist activity were largely avoided to focus on Berlin citizens.

#### Questionnaire and stimuli

The questionnaire was composed of three parts and included photographic stimuli that depicted *Ailanthus* in different urban contexts (Fig. 2; the original master version is provided as Suppl. material 1). The first part of the questionnaire assessed respondents' knowledge of *Ailanthus* in two ways since valuations of non-native species can be modu-

(B) Group of trees in greenspace

### (A) Tree in park



**Figure 2.** Photographic stimuli depicting *Ailanthus altissima* in different urban contexts in Berlin **A** as a single tree in a park **B** a group of trees in a green space along a road **C** a wild tree in tree pit, and **D** wild trees along an urban rail line. The urban settings thus show a gradient from designed to wild settings.

lated by familiarity with the species (Sharp et al. 2011; Luna et al. 2019). Respondents were first asked whether they recognize the tree (self-estimated knowledge) and second if they were able to provide its correct colloquial or botanical name (assessed knowledge).

The second part of the questionnaire assessed respondents' preferences for *Ailanthus* in four urban contexts by asking "How do you like the tree in this situation?" using a five-point Likert scale (1, [like] not at all – 5, [like] completely) and showing different photographic scenes (Fig. 2). Likewise, in this part of the questionnaire we assessed on the same five-point scale how respondents accepted three different management options that represent major approaches in managing non-native plant species (Sharp et al. 2011; i.e. 'leave alone', 'adaptive on-site' and 'complete removal' management). These strategies were addressed by using easily accessible wording by asking: "how should this tree be handled in Berlin?", with the answers "let it grow everywhere" ('leave alone' management), "remove it only if problems exist" ('adaptive on-site' management), and "remove it everywhere" ('complete removal' management).

In the third part of the questionnaire we assessed the sociodemographic background of the respondents (Table 1) since sociodemographic variables (i.e. age and gender; Shackleton 2019c) are often related to the acceptability of management measures. Further, 'close to nature' behaviour and attitude (gardening, visits to urban green areas and role of nature) are important factors, when it comes to how people value urban biodiversity in different urban situations (Fischer et al. 2018).

To test whether respondents' preference ratings for different management strategies were motivated by xenophobia we used two versions of the question on management strategies. In the first version we asked: "how should this tree be handled in Berlin?" The second version provided additional information (i.e. provided knowledge) by changing "this tree" to "this non-native tree from China". We expected that interviewees with a xenophobic worldview would prefer the 'complete removal' strategy significantly more than other respondents after they had gained the information on the tree's non-native status in Germany due to its Chinese origin.

Five different photographs of Ailanthus were used as photographic stimuli in the questionnaire. In the first part, the photograph showed a single and mature tree within a typical background (i.e. urban street, in front of an apartment building) to ask participants whether they recognize the species (self-estimated knowledge) and could provide its name (assessed knowledge). In the second part, four photographs were used to assess the preferences of Ailanthus in four ubiquitous urban situations, which span a gradient from intensively designed green spaces to situations in which Ailanthus thrives as a wild tree outside of green spaces (Fig. 2). The first of these showed Ailanthus as a mature, cultivated tree in a traditional urban park. The second photograph showed a group of tall trees representing a small patch of a likely wild-grown emerging forest that had been incorporated into a traditionally managed green space along an urban street. The third depicted *Ailanthus* as a young, wild tree associated with a fenced cultivated tree in a streetscape. The last photograph showed a group of young, wild-grown trees along an urban rail line. All photographic stimuli represented situations at human eye level and field of vision with similar light conditions and flat topographic structures without aspects that might bias vegetation evaluations such as humans, animals, litter, or open water.

# Statistical analyses

We fitted ordinal logistic models using the 'polr' command from the MASS package in R version 4.0.2 (R Core Team 2020). Our response variables were contextdependent preferences for the *Ailanthus* in four urban contexts and acceptability of three management strategies as illustrated in Fig. 1. For each candidate model (i.e. four for preferences and three for management strategies), we included the following explanatory variables separately as fixed effects (see Table 1 for details): the first set of models included (a) different forms of knowledge (self-estimated knowledge, assessed knowledge, provided knowledge (the latter only for acceptability of management strategies)) as explanatory variables; the second set of models included (b) 'close to nature' behaviour and attitude (gardening, visits to urban green areas and role of nature) as explanatory variables; the last set of models included (c) three sociodemographic variables (age, gender and professional context) as explanatory variables.

To take into account that gender and professional context or age and professional context potentially interact in their effect on responses towards *Ailanthus*, we tested interaction terms between age, gender and professional context. In detail, we used responses towards *Ailanthus* (i.e. preferences or acceptability) as response variables and interaction terms between age, gender and professional context as explanatory variables. There were no significant interactions between these variables, i.e. no indication of a relationship between 'age', 'gender' or 'level of expertise' and the response variables. Therefore, we included each variable separately as fixed variables in the final models.

# Results

In total, we surveyed 197 participants of which 101 were laypeople and 96 were practitioners. Most respondents were male (58%, while 40% were female and 2% diverse or did not mention their gender; hence too few in numbers for the statistical analysis), younger (48% of the respondents were under 30 years, while 30% were between 30 and 60 and 19% were above 60 years old; 3% did not mention their age) and born in Germany (88% of respondents, while the remaining participants were born outside Germany or did not mention their place of birth).

Age, class and gender distribution were different among laypeople and practitioners (Table 2). The number of younger people (< 30 years old) was higher in the practitioner compared to the laypeople group. Congruently, there were more people aged 30 years or older in the laypeople group compared to the practitioner group (x-squared = 72.8, df = 2, p < 0.001). Similarly, gender was not equally distributed. There were more males and fewer females in the practitioner group compared to the laypeople group (x-squared = 29.5, df = 2, p < 0.001). Since there was no indication of an interaction between gender, age and professional background (i.e. calculating interaction terms in our models), we considered age, gender and professional background separately in our further analyses.

#### Knowledge of Ailanthus

Overall, 83% of the respondents mentioned that they recognised *Ailanthus* when they saw it on the photograph (i.e. self-estimated knowledge). However, only 26% mentioned its correct colloquial or botanical name (i.e. assessed knowledge). Comparing the self-estimated knowledge between practitioners and laypeople, we found that practitioners (mean = 0.92, SD  $\pm$  0.28) mentioned significantly more often than laypeople (mean = 0.73, SD  $\pm$  0.45) that they would recognise the tree on the photograph (*p* < 0.01). In relation to assessed knowledge, practitioners (mean = 0.45, SD  $\pm$  0.50) also provided significantly more often the correct name of *Ailanthus* compared to laypeople (mean = 0.03, SD  $\pm$  0.16) (*p* < 0.001).

	Practitioners	Laypeople
Age		
< 30 years (Category 1)	72	22
30-60 years (Category 2)	18	42
>60 years (Category 3)	0	37
Gender		
Male	73	42
Female	20	59
Diverse	2	0

Table 2. Number of practitioners and laypeople distributed across age and gender categories.

#### Preferences in different urban contexts



**Figure 3.** On the left, overall preference ratings of respondents for *Ailanthus altissima* in four different urban contexts (see Fig. 2). Different letters indicate significant differences (Tukeys HSD p < 0.001 for a to c, a to d, b to c and b to d, p < 0.01 for a to b and p < 0.05 for c to d). On the right, preferences are differentiated between practitioners and laypeople.

# Preferences of *Ailanthus* in different urban contexts and predictors for these preferences

Overall, respondents reported a broad range of preferences for the photographs showing *Ailanthus* in different urban contexts (Fig. 3). Most respondents preferred the cultivated, mature tree in the urban park (mean = 4.5, SD = 0.7), followed by the group of trees in the green space along the road (mean = 4.1, SD = 1.0). The responses to the photographs showing younger, wild populations were less positive. On average, respondents liked the wild trees along the rail line to some extent (mean = 2.8, SD = 1.3) and the wild *Ailanthus* in the tree pit the least (mean = 2.4, SD = 1.3). These preferences for *Ailanthus* significantly differed between the contexts depicted in the photographs (ANOVA, F (3, 770) = 150.7, p < 0.001). In detail, *Ailanthus* was significantly more preferred in situations showing tall trees in the two green spaces (park, along urban street) compared to the scenarios depicting young, wild trees along the rail line and in the tree pit (Tukeys HSD, p < 0.001). Further,

**Table 3.** Predictors of respondents' preferences of *Ailanthus* in different urban contexts. Parameter estimates are derived from ordinal logistic regression. Significance levels shown in bold and with asterix for \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001.

	Tree in park	Group of trees in green space	Wild in tree pit	Wild along rail line
-	Estimate (S.E.)	Estimate (S.E.)	Estimate (S.E.)	Estimate (S.E.)
a) Knowledge				
Self-estimated knowledge	-0.25 (0.47)	0.02 (0.45)	0.01 (0.41)	0.43 (0.41)
Assessed knowledge	-0.69 (0.35)*	-0.50 (0.33)	-0.79 (0.34)*	-0.53 (0.33)
b) 'Close to nature' behaviour and atti	itude			
Gardening	-0.24 (0.34)	0.05 (0.30)	-0.51 (0.31)	0.15 (0.30)
Visiting green areas	-0.01 (0.18)	0.06 (0.17)	0.23 (0.18)	0.06 (0.16)
Role of nature	0.97 (0.22)***	0.33 (0.21)	1.01 (0.23)***	0.89 (0.22)***
c) Sociodemographic variables				
Laypeople (compared to practitioners)	1.99 (0.45)**	0.88 (0.37)*	1.33 (0.37)***	0.68 (0.35)
Age (compared to <30 years)				
Between 30 and 60 years	-0.55 (0.42)	-0.75 (0.36)	1.00 (0.35)**	0.54 (0.34)
Older than 60 years	-0.78 (0.58)	-0.59 (0.46)	-0.03 (0.43)	0.09 (0.43)
Gender				
Female (compared to male)	0.71 (0.34)*	0.27 (0.30)	0.65 (0.29)	0.06 (0.29)

preferences also differed within these contexts, with a higher preference for the single mature tree in the urban park than the group of mature trees in the green space along the road (Tukeys HSD, p < 0.01). The wild trees along the rail lines were significantly more preferred than the wild tree in the tree pit (generally the least preferred context) (Tukeys HSD, p < 0.05).

Overall, a wide range of preferences also existed for the practitioners and laypeople for photographs depicting *Ailanthus* in various urban contexts (Fig. 3, bar charts at the right). In our models, professional context, role of nature, assessed knowledge, gender and age had some predictive potential on respondents' preferences of *Ailanthus* in the four urban contexts (Table 3). First, laypeople preferred *Ailanthus* in three out of the four contexts (i.e. except rail lines) significantly more compared to practitioners. Second, respondents for which nature plays a large role when they visit urban green areas, also preferred *Ailanthus* in three out of the four contexts (i.e. except 'group of trees in green space') significantly more than other respondents. Third, assessed knowledge was a negative significant predictor for preferences of *Ailanthus* in urban parks and in tree pits. Last, as for gender and age, female respondents preferred seeing *Ailanthus* in urban parks significantly more than male respondents and respondents between 30–60 years preferred seeing wild grown *Ailanthus* in tree pits significantly more than younger respondents (under 30 years old).

# Acceptability of management strategies for *Ailanthus* and predictors for this acceptability

Respondents' support of management strategies significantly differed between the three suggested strategies (ANOVA, F (2, 558) = 205.1, p < 0.001; Fig. 4). The 'adaptive on-site' management strategy yielded the most support (mean = 4.0, SD = 1.2) and was



**Figure 4.** On the left, acceptability of three management strategies on *Ailanthus*. Significant differences shown by letters (Tukeys HSD p < 0.001 for a to b and c as well as b to c). On the right, preferences are differentiated between practitioners and laypeople

**Table 4.** Predictors of respondents' acceptability of management strategies for *Ailanthus*. Parameter estimates are derived from ordinal logistic regression. Significance level shown at shown in bold and with asterix for \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001.

	Leave alone	Adaptive on-site	Complete removal
	Estimate (S.E.)	Estimate (S.E.)	Estimate (S.E.)
Knowledge			
Self-estimated knowledge	-0.83 (0.42)*	0.10 (0.43)	0.16 (0.53)
Assessed knowledge	-1.07 (0.37)**	-0.53 (0.35)	0.62 (0.41)
Provided knowledge	-0.04 (0.33)	-0.34 (0.32)	0.12 (0.38)
'Close to nature' behaviour and attitude			
Gardening	-0.15 (0.31)	-0.50 (0.31)	-0.23 (0.35)
Visiting green areas	-0.18 (0.17)	-0.01 (0.17)	0.01 (0.19)
Role of nature	0.76 (0.22)***	0.25 (0.20)	-0.64 (0.23)**
Sociodemographic variables			
Laypeople (compared to practitioners)	1.38 (0.37)***	1.23 (0.39)**	-1.29 (0.43)**
Age (compared to <30 years)			
Between 30 and 60 years	0.10 (0.35)	-0.81 (0.37)*	0.09 (0.41)
Older than 60 years	0.91 (0.45)*	-1.40 (0.49)**	-0.39 (0.63)
Gender			
Female (compared to male)	0.55 (0.30)	-0.22 (0.30)	0.15 (0.36)

significantly more accepted than the other two management strategies (Tukeys HSD, p < 0.001). Respondents moderately agreed with the 'leave alone' management strategy (mean = 2.7, SD = 1.5), which involves renouncing management. The 'complete removal' management strategy gained the least support and was on average rejected (mean = 1.5, SD = 0.9) (Fig. 4).

Professional context, role of nature, age, self-estimated knowledge and assessed knowledge had predictive potential on the acceptability of the three management strategies (Table 4). First, laypeople accepted the most severe management strategy, 'complete removal', significantly less than practitioners, while they accepted the re-

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maining two management strategies, 'leave alone' and 'adaptive on-site' management, significantly more than practitioners (Fig. 4, bar charts at the right). Second, respondents for which nature plays a large role when they visit urban green areas accepted the 'complete removal' strategy significantly less and the 'leave alone' strategy significantly more than other respondents. Additionally, self-estimated and assessed knowledge had negative predictive potential on the 'leave alone' management strategy while provided knowledge on the non-native status of *Ailanthus* had no predictive potential on any management strategy. As for age, older people (above 60 years old) significantly preferred the 'leave alone' strategy more than younger respondents. Respondents between 30 and 60 and those older than 60 years accepted 'adaptive on-site' management strategy significantly less than younger respondents. Gender had no predictive potential on any of the three management strategies.

# Discussion

How citizens view widespread invasive species in different urban settings is largely unknown. We thus investigated (i) urban residents' preferences on the invasive tree species *Ailanthus altissima*, shown as a cultivated or wild tree in multiple urban situations in Berlin, (ii) which management strategies respondents considered acceptable towards *Ailanthus*, and (iii) how respondents' sociodemographic background and 'close to nature' behaviour and attitude predicted their preference ratings. Major insights of our quantitative survey were:

(1) It is not the identity of an invasive species alone that matters for urban residents' preferences, but the context in which it thrives. The respondents' view of *Ailanthus* was diverse, with significant differences between each of the urban contexts in which the species was shown. Citizens' view on a widespread invasive species is thus clearly context dependent.

(2) Respondents expressed the most support for adaptive on-site management of Ailanthus, some support for leaving it alone, and the least support for complete removal. This suggests that most respondents in our study generally accept *Ailanthus* as part of the urban environment and at the same time support management efforts in specific problematic situations.

(3) The views on Ailanthus and adequate management strategies depended on respondents' sociodemographic backgrounds and their 'close to nature' behaviour and attitude. Practitioners were more critical about *Ailanthus* than laypeople. Respondents with a 'close to nature' behaviour and attitude had a more positive view on *Ailanthus* than others and expressed more support for leaving it alone on urban sites. This indicates that biophilia in urban societies can also cover widespread invasive species. These insights have important implications for environmental policies and management plans on *Ailanthus* in urban regions.

### Preferences of Ailanthus depend on the urban context

Approval or disapproval of non-native plants depend on people's general value system, which usually varies within different groups of society (Estéban et al. 2015; Shackleton et al. 2018), and largely differ for different species considered (e.g. Lindemann-Matthies 2016). These differences may be related to the fact that both positive and negative impacts may be attributed to different, or even the same species (e.g., Potgieter et al. 2019a; Shackleton and Mograbi 2020). As a consequence, a considerable amount of respondents in previous studies have been shown to tolerate some invasive non-native plant species in cities (Potgieter et al. 2019a). Yet previous studies largely explored residents' views in regard to the species itself, i.e. to species identity, and thus yielded insights into general approval or disapproval of urban residents for a given species. Our study goes one step further by assessing how respondents' preferences for the same species depend on different urban contexts, spanning from intentionally planted to wild Ailanthus trees. The significantly different preference ratings on each of the four shown situations reveal: peoples' views on a widespread invasive species not only depend on its identity and related features - such as beauty (Lindemann-Matthies 2016), charisma (Jarić et al. 2020), or usefulness (Shackleton and Mograbi 2020) - but also on the context in which it thrives. Citizens in Berlin thus tolerate, or even appreciate, cultivated or wild Ailanthus in some contexts while disapproving of it in others.

The respondents clearly liked Ailanthus in the two settings that showed tall Ailanthus trees in green spaces, but significantly less in the two wild settings with younger individuals (Fig. 3). This major difference can be explained with the presence, or absence of "cues to care" (Nassauer 1995), an important issue in landscape perception or preferences (Li and Nassauer 2020). The highest preferences for green space settings can be related to three mechanisms that underlie the significance of "cues to care" for landscape preferences (Li and Nassauer 2020), i.e. (i) *immediate recognisability*: the tall trees are easily recognizable as elements of traditional green spaces; (ii) human presence or intention: the size of the trees and their association with traditionally designed green spaces indicate that they are intended green space components; (iii) cultural traditions or social norms: both the individual tree and the group of trees are traditional design elements of English landscape gardens and their ubiquitous urban equivalents. Our study thus indicates that Ailanthus trees are broadly accepted by urban residents when integrated into traditional urban green spaces. However, in this regard also the downside of displaying various urban contexts (wild to designed) has to be considered: our photographs were not standardized with regard to environmental settings such as an even blue sky, small-scale urban infrastructures such as benches or fences, light settings or green space management (e.g., manicured lawns) that could have potentially biased the preference ratings.

The photos showing wild *Ailanthus* plants received significantly less favourable ratings than the contexts with mature trees in green spaces. This may be due to the recognisability of wild populations as unintended elements in urban open spaces. They represent wilderness components resulting from, and clearly indicating, the functioning of unmanaged, natural processes in designed environments (Kowarik 2018). In

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this way, the wild context does not match the deeply rooted cultural traditions in maintaining neat open spaces. Wild, near-natural vegetation is still more highly valued by people that assign nature-related values to such scenarios in an urban green space context (Lampinen et al. 2021).

Yet the ratings of the wild scenes were not consistently negative. About 53% of the respondents liked the wild rail line situation "very much", "mostly", or "to some extent" opposed to 46%, which liked it "little" or "not at all" (Fig. 3). Thus, about half of the respondents shared a rather positive view on these wild populations. This share is similar to that of "wildness enthusiasts", opposed to people preferring highly maintained green spaces, in a study on preferences for wild vs. maintained roadside vegetation in Berlin (Weber et al. 2014). Respondents' preference ratings for the wild Ailanthus along the rail line could indicate a change in cultural values, leading to an increased valuation of wild urban nature. Indeed, the integration of wild urban nature is a current topic in urban development and design (Kühn 2006; Del Tredici 2010; Mc-Kinney et al. 2018; Hwang and Yue 2019), with Berlin being a forerunner in this area. Since the late 1980s, new types of wild urban nature such as naturally re-vegetated vacant land have been integrated into the city's green system (Lachmund 2013; Kowarik 2018, 2019). Respondents from Berlin could thus be more familiar with components of wild urban nature than people from other cities. Previous studies on landscape preferences revealed considerable support for wild urban vegetation (e.g. Weber et al. 2014; Fischer et al. 2018; Bonthoux et al. 2019; Hwang et al. 2019; Li et al. 2019). The survey at hand indicates that a widespread invasive tree species can receive a very high level of acceptance when integrated into green spaces, while respondents' view on wild Ailanthus trees is less favourable, but not consistently negative.

Preference ratings also differed significantly among the two wild situations, with less support for the tree pit context than the rail line (Fig. 3). The former could easily be read as a situation in which wild *Ailanthus* could conflict with an intended element of the green infrastructure: the carefully fenced young street tree. Respondents could have expected negative effects on the planted tree due to competition from the wild tree. Alternatively, but not mutually exclusive, the wild tree in the tree pit situation could be perceived as an indicator of neglect in a traditionally highly controlled urban environment. In contrast, the scene with the wild *Ailanthus* along the rail line might be read as re-vegetation of a harsh urban site, which might be associated with beneficial environmental effects but without competition with other species.

# Broad acceptance of adaptive on-site management

Wild populations of *Ailanthus* may challenge the traditional aesthetic ideal of tidy open spaces and the required technical efforts to maintain urban green spaces, transportation corridors or built structures (Kowarik and Säumel 2007; Sladonia et al. 2017). Due to the high abundance of *Ailanthus* in Berlin, respondents were likely aware of the non-native species and related problems. Surprisingly, 31 of the 138 respondents that knew *Ailanthus* before (based on 'self-estimated' knowledge)–and generally 51 of all 197 respondents-were in favour (i.e. ratings '4' and '5') of the 'leave alone' manage-

ment strategy and only a minority of respondents (ca. 8%) supported the 'complete removal' of *Ailanthus* (Fig. 4). Since we did not ask respondents about perceived threats due to *Ailanthus*, missing knowledge about possibly associated problems could also explain the support for the 'leave alone' strategy.

A broad majority of respondents, however, supported the 'adaptive on-site' management strategy, and thus seemingly agreed to action if problems are evident in specific cases. This adds evidence to previous studies that also revealed the most support for an intermediate position between doing nothing and completely removing widespread invasive species (Sharp et al. 2011). A study from Austria, for example, found broad support for invasive species management, but less support for the use of lethal measures or herbicides (Höbart et al. 2020). In a study from Cape Town, South Africa, respondents broadly supported control measures, but the majority did not recognize a high management priority (Potgieter et al. 2019a). The clear support for 'adaptive onsite' management in our study corresponds well with the respondents' preferences for Ailanthus since these clearly depended on the urban context. Most respondents seemed to tolerate, or even appreciate Ailanthus in some situations, while disapproving of it in others. Overall, our study thus indicates a lack of support for a total ban (or removal) of Ailanthus in Berlin, but a high level of acceptance for problem-oriented management. Which kind of problems would justify the application of on-site management from the respondents' view or how the specific context relates to the approval or disapproval of management strategies should be analysed by a follow-up study.

#### What explains people's views on Ailanthus?

Our survey shed light on urban residents' views on *Ailanthus* as a widespread invasive tree species in Berlin, including the tree itself in different urban contexts and potential management strategies. We could not ask about the motivation behind respondents' preference ratings and acceptability due to the required brevity in interviews in the field. However, we related sociodemographic background and 'close to nature' behaviour and attitude of the respondents to their preference ratings and the acceptability of management strategies, which allowed us to describe these relationships in some detail.

# Role of knowledge

As expected, practitioners were generally more critical towards *Ailanthus* than laypeople, with less favourable preference ratings in the urban contexts (except the rail line situation) and a higher support for the 'complete removal' strategy. This is in line with other studies revealing that respondents with a formal training in environmental issues are more aware of invasion risks and support more aggressive management strategies like attempt eradication (Luna et al. 2019; Cordeiro et al. 2020; Nguyen et al. 2020). Practitioners in our study were likely aware of management problems related to *Ailanthus* due to their experience in urban landscaping and green space maintenance.

However, a considerable share of practitioners also expressed positive ratings on the urban contexts and on the less strict management strategies (Figs 3, 4). Despite

clearly pronounced differences between laypeople and practitioners, the expert view on *Ailanthus* in cultivated and wild urban settings was not uniformly negative. This indicates that practitioners might not only be aware of risks, but also of some environmental benefits that can be supported by both cultivated and wild *Ailanthus* trees in urban environments (Kowarik and Säumel 2007; Sladonia et al. 2017; see below).

Since an enhanced level of knowledge was related to a higher sensitivity towards invasive species in other studies (e.g. Sharp et al. 2011; Shackleton and Shackleton 2016), we analysed how different levels of knowledge on Ailanthus related to respondents' preference ratings. The self-estimated knowledge, indicated by interviewees' response on recognizing the species, was not related to any preference rating in the four urban contexts. However, respondents that assigned the correct name to Ailanthus ('assessed knowledge') expressed less favourable ratings on two of the four urban scenes: the individual mature tree in the park and the young, wild tree in the tree pit. Both, selfestimated and assessed knowledge were negatively related to preferences on the 'leave alone' strategy. This indicates that people who had shared some knowledge on Ailanthus (analogously the practitioners in the practitioners-laypeople comparison) were less willing to let it grow without any intervention. Interestingly, 'provided knowledge' did not show any effect on the acceptability of management strategies. While other studies have shown the effect of information on the acceptability of management strategies (Ford et al. 2009) or landscape preferences (Straka et al. 2016), it is likely that illustrating the non-native status of the tree in Berlin by including information about its Chinese origin was not enough information for a shift of the acceptability of management strategies in this study.

# Age and 'close to nature' behaviour and attitude

Preference ratings for the urban scenes were only weakly and inconsistently related to age and gender. However, older people (> 60 years) were significantly more willing to accept *Ailanthus* than younger people. In other studies, though, older respondents tended to perceive invasive plants more negatively than younger people (Potgieter et al. 2019a; Nguyen et al. 2020). These contrasting results may depend on the identity of the addressed species, on respondents' familiarity with it or on the urban settings in which the species grows.

Respondents' nature-related activities such as visiting green spaces, which were important predictors of preference ratings in other studies (Fischer et al. 2018), were not significantly related to any of the outcomes. However, respondents who said that nature plays an important role when visiting green spaces liked three of the four urban contexts significantly more than other respondents. Consistently, this close to nature attitude was positively related to the 'leave alone' strategy, and negatively to the 'complete removal' strategy. In a study from Cape Town, respondents with a higher level of environmental awareness were more likely to perceive non-native species as beneficial, but were also more supportive than others of control measures to protect biodiversity (Potgieter et al. 2019a). Our results suggest that *Ailanthus* is less likely to be perceived as a threat to biodiversity in Berlin. Our study indicates that the biophilic view that exists in urban societies (Wilson 1984; Beatley 2010) can also cover widespread invasive species despite apparent challenges such as an increased demand for maintenance. This conclusion is also supported by the highest acceptance of the 'adaptive on-site' management strategy, as this approach explicitly excludes general measures against the species independently from the situation in which it thrives.

#### Missing indication of xenophobia

Environmental preferences not only rely on knowledge about the addressed issue but also on values and beliefs (Ives and Kendal 2014). We tested here whether respondents' preference ratings for different management strategies regarding a widespread invasive tree species were underlain by xenophobia, a controversial issue in invasion studies (Richardson and Ricciardi 2013). We expected that interviewees with a xenophobic worldview would prefer the 'complete removal' strategy significantly more than other respondents after they had gained the information on the non-native status and Chinese origin of *Ailanthus*. This approach seemed appropriate since we did not otherwise inform the interviewees about the origin or invasive status of *Ailanthus*, or associated problems, to avoid a bias in the preference ratings due to a priori information.

The lack of differences between the answers to the two versions of the question indicates that preference ratings were primarily related to respondents' views on *Ailan-thus* – independently from their knowledge of its non-native status or origin. Alternatively, the absence of differences might reflect that the additional information did not increase respondents' knowledge, if they were previously aware of its non-native status. This explanation, however, is not supported by the gap between the self-estimated and assessed knowledge: 83% of all participants reported recognizing the tree, but only 26% knew its correct name. This indicates that a large majority of respondents did not have deeper information about *Ailanthus*, such as its non-native status, although they were likely familiar with the tree due to its abundance in Berlin for about 40 years (Kowarik and Böcker 1984). This supports the interpretation of our results as an indication of lacking xenophobia in respondents' views on *Ailanthus*.

# Implications

Our results have implications for the implementation of management measures on *Ailanthus*, which have to be established according to Article 19 of the EU Regulation on invasive alien species (European Parliament 2014). Brundu (2017) highlighted the need of considering human values in the design and implementation of such measures. He also identified a lack of more detailed studies on the views of different stakeholders on *Ailanthus* in cities, although the inclusion of such views is essential for the success of management measures. Our study revealed that citizens' views on *Ailanthus* were context dependent, partially related to the background of respondents, and ranged from approval to disapproval. These results thus do not support the assumption that "*Ailanthus* invasions

are generally perceived as a problem in the habitats and land uses where *Ailanthus* stands are established and costly control intervention are often occurring" (Brundu 2017). Even in Berlin, as an *Ailanthus* hotspot, urban residents did not generally perceive this species as a problem. Consistently, respondents clearly supported the 'adaptive on-site' strategy.

Controlling *Ailanthus* only when concrete, on-site problems exist seems to be an appropriate strategy for cities like Berlin. *Ailanthus* largely spreads in the vicinity of female seed trees (Paź-Dyderska et al. 2020). It should thus not be assumed that *all* urban trees (both male and female) are a *general* threat to areas or species of conservation concern that are usually located in the urban fringe or surroundings of cities. Risks may emerge, however, when female trees are close to conservation areas or as a result of exceptional seed dispersal over very long distances. In such cases, the removal of female trees can be useful, for which a suite of control measures is available (Brundu 2017). These control approaches can also be used when *Ailanthus* becomes a nuisance in urban open spaces. However, this often requires long-term maintenance, as individual trees and clonal populations can regenerate vegetatively very well (Kowarik and Säumel 2007). Moreover, individual methods such as the application of herbicides or pathogenic fungi are not permitted everywhere, including Berlin.

Our results indicate that a management plan of *Ailanthus* could meet acceptance under the condition that the included measures are tailored to manage specific situations (e.g. removal from nature reserves and the management of propagule sources in the vicinity of susceptible habitats of conservation concern). However, the considerable share of respondents that preferred a general hands-off strategy indicate how important communication strategies are to justify management approaches and to explain their implementation.

Another implication is on the integration of *Ailanthus* in urban greening. Given the abundance of cultivated and wild trees in Berlin, as well as in many cities, a complete removal of the species is not realistic due to its regeneration potential and would not receive support from residents as indicated by our study. Management plans should thus focus on counteracting or preventing evidenced conflicts at the local scale and prevent invasions of habitats of conservation concern. According to the EU Regulation on invasive alien species, such measures should also "be proportionate to the impact on the environment and appropriate to the specific circumstances of the Member States, [and] be based on an analysis of costs and benefits" (European Parliament 2014).

The classic challenge here is to balance the negative and positive effects that can be associated with invasive species (van Wilgen 2012; Potgieter et al. 2019b; Shackleton et al. 2020; Brundu et al. 2020; Vimercati et al. 2020). Indeed, a range of services and disservices have been reported for urban *Ailanthus* trees (Kowarik and Säumel 2007; Sladonia et al. 2017): *Ailanthus* is well adapted to climate warming, and thus to the urban heat island (Roloff et al. 2019); it further supports a range of regulating ecosystem services, which have been quantified for some cities (e.g., air pollution removal, storm water management, carbon sequestration; Kim et al. 2015; Kim 2016; Riley et al. 2018; Arrington 2020). Our study adds the insight that both cultivated and wild trees can support cultural ecosystem services in cities as indicated by the considerable share of positive preference ratings for some urban contexts.

This leads to the conclusion that urban management plans on *Ailanthus* should combine three aims: (i) to perform on-site management in case of evidenced problems; (ii) to prevent the invasion of susceptible habitats of conservation concern and contain urban populations when feasible; and (iii) to develop and test novel approaches of integrating wild *Ailanthus* trees deliberately into the urban green infrastructure – if risks for conservation areas can be excluded. We thus argue for multidirectional management approaches towards *Ailanthus* in urban regions.

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# Supplementary material I

#### Original questionnaire in German language

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Data type: questionnaire

- Explanation note: This version shows the master version, from which 4 subversions were generated: (a) without the explanation on the tree's origin, (b) with the explanation on the tree's origin and an additional question on practitioner's working environment, (d) without the explanation on the tree's origin and an additional question on practitioner's working environment.
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RESEARCH ARTICLE



# Landscape restoration due to Xylella fastidiosa invasion in Italy: Assessing the hypothetical public's preferences

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#### Abstract

Since 2013, the olive landscapes have gradually degenerated due to the spread and establishment of *Xylella fastidiosa* subsp. *pauca* (hereafter *Xf*) in Apulia, southern Italy. From 2013 to 2019, a total of approximately 54,000 hectares of olive orchards in the south of this region have been seriously damaged, and their restoration will progressively regenerate the economic, social, cultural and environmental nonmarket benefits. Since there is a willingness to restore the affected landscape in the best interest of the local citizens, this research aims to predict their preference heterogeneity and willingness to pay (WTP) to improve this landscape and continue research and experimentation in relation to Olive Quick Decline Syndrome Disease by the bacterium. For this purpose, a choice experiment method is used. The social field survey includes a representative sample of 683 respondents in three major cities (Foggia, Bari and Lecce) of Apulia region. The results reveal that for the local citizens interviewed, the most appreciated olive landscape services are cultural heritage and aesthetic values. In addition, the findings revealed citizens' positive appreciation of improving the damaged olive landscape, while respondents are not willing to pay a premium for research.

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The results show that the average value that Apulians are willing to pay for landscape restoration is about 5.7 million of  $\in$  per year. Further, this research has implications for land use planners in the study area, which faces issues of harmful pathogen management and land revival.

#### **Keywords**

alien species, biological invasion impact, choice experiment, economic costs, ecosystem services, environmental changes, social perception, willingness-to-pay

# Introduction

Olive-growing is recognized as a multifunctional ecosystem in Italy. It shapes the landscape of the countryside with a particularly visual spatial representation (Lanfranchi and Giannetto 2012). Furthermore, this aesthetic value is not only the absolute nonmarket feature of this ecosystem, but provides considerable socio-cultural services (Severini 2006), economic effects (Viganò 2006), ecological benefits (Bernetti et al. 2006; Torquati et al. 2006), and conservation of the agro-biodiversity (Corrado et al. 2011; Fernández-Habas et al. 2018). However, since 2013, a part of this rural landscape has increasingly lost these attributes in the Salento Peninsula of Apulia in southern Italy. Consequently, a total of approximately 22 million plants have been affected and approximately 6.5 million olive trees died (Beck et al. 2019), causing real damage to the landscape and identity of the study area.

The loss is due to Olive Quick Decline Syndrome Disease (OQDS, previously known as "CoDiRO", recently named "De Donno") (Saponari et al. 2013, 2018) caused by Xylella fastidiosa subsp. pauca (hereafter Xf), a quarantine plant pathogen for the EU area, where stringent and specific regulations apply. So, Apulian olive trees of Lecce province were affected by Xf and characterized by leaf scorching, scattered desiccation of twigs and branches conferring thus a burned aspect and subsequent tree mortality (Saponari et al. 2013) as shown in Pictures 1, 2. The most severely and impressively affected olives are the centuries-old trees of the local highly susceptible cultivars Cellina di Nardò and Ogliarola salentina which marked by (OQDS) from incipient signs of infection to plant death. Monumental trees with a skeletal appearance, severely pruned to promote new vegetation, have shown dramatic decline and severe symptoms of desiccation where the few shoots produced are already dead. In the field, remarkable differential response to natural infection by Xf (Giampetruzzi et al. 2017) was revealed in adjacent rows of olive trees of cultivars Leccino and Ogliarola salentina and comparative analysis of the transcriptome of these varieties was carried out to investigate the reasons for this differential behavior (Giampetruzzi et al. 2016). Moreover, in experimental fields and controlled conditions for the evaluation of resistance/susceptibility of the varieties/species, through mechanical inoculation with bacterial cultures, strain "De Donno", does not infect grapevines and citrus whereas it multiplies readily in oleander, olive seedlings and rooted cuttings of cv. Cellina di Nardò and to a much lesser extent in other olive cultivars like Coratina, Frantoio, Leccino (European Food



Picture 1. Olive leaf scorching due to Xylella fastidiosa invasion in Apulia region. Source: Infoxylella.



Picture 2. Olive trees mortality caused by Xylella fastidiosa in Apulia region. Source: Infoxylella.

Safety Authority 2015; Saponari 2016). However, one of the best solutions was the search for resistant varieties which are a highly desirable approach with the aim of the possible reconstitution of the Salentinian olive industry based on a set of cultivars that can replace for the largely predominant and highly susceptible ones. Leccino and FS-17 have already been identified and a patch-graft with these resistant varieties on old infected olive trees was piloted, considering this cultural practice as an encouraging starting point in the research for resistant material.

This bacterium (Sequence Type 53) originating on coffee plants in Costa Rica is driven by an insect-vector, *Philaenus spumarius*, which is widely distributed in Apulia region (Bosco 2014; European Food Safety Authority 2015; Martelli et al. 2015). Further, Frem et al. (2020) predicted that this bacterium may invade new European and Mediterranean countries. However, the bacterium was also detected in *Neophilaenus campestris* and *Euscelis lineolatus* indicating the potential vectoring roles of these insects for the spread of the bacterium in Apulian region (Elbeaino et al. 2014) while the spittlebug *P. spumarius* was the most abundant species found in orchards on both weeds and olive trees in Italy (Cornara et al. 2017). To better understand the role of the host plant on the ecology of the infections, numerous plant species have been shown to be colonized by *Xf* in southern Apulia, including almond, oleander, cherry and other species (Saponari et al. 2013, 2014). This may confirm the impacts of host plant species on the efficiency of transmission of *Xf* by *P. spumarius* and therefore the ability of the bacterium to spread rapidly and trigger an outbreak. *Xf* colonizes the xylem network of the trees (Wells et al. 1987).

As a consequence, by destroying the rural landscape in the study area, Xf is gradually inducing changes in olive trees landscape which provides a set of economically valuable goods and provisioning services such as food diet (olive and olive oil), regulating services that affect climate, biodiversity, sustainable agriculture and cultural services that provide recreational, aesthetic, and educational and research features. Thereby, these benefits contribute towards daily life and human well-being in terms of health, security, good life and social relations. Ultimately, linking these attributes to human wellbeing constitutes an integral part of the economic analysis that is applied when decisions are necessary for the concerned stakeholders to manage a biological invasion process (Emerton and Howard 2008). In this framework, the Figure 1 highlights the links between Xf invasion, olive landscape services and public's well-being. Changes in ecosystem services and human well-being, give rise to assess the public's preferences about landscape recovery.

Furthermore, the provision of ecosystem services is not perceived or observed similarly by all social groups (Sardaro et al. 2016a). Therefore, assessing their preferences about ecosystem recovery such as olive landscape restoration constitutes an integral part of the economic analysis of invasive species that is applied when policy decisions should be taken to manage biotic invasions (Emerton and Howard 2008). Moreover, Apulian residents as taxpayers would pay additional costs for landscape recovery and rehabilitation. Meanwhile, residents' preferences or opinions must be taken into consideration in the decision-making process (Haltia 2015) in order to comprehensively assess landscape (Tagliafierro et al. 2013). Without this consideration, the prevention of wasteful and imprudent resource allocation cannot be guaranteed. Given this, this research aims to provide an assessment of local citizens' preferences for different olive landscapes changes and for pursuit of research associated to *Xf*. Also, the study provides their WTP for the improvement of the damaged olive landscape in Apulia within an economic analysis of biotic invasive framework. Burgess et al. (2012) points out that citizens' preferences in relation to a non-excludable and non-rivalrous environmental



**Figure 1.** Overview of the impact of *Xylella fastidiosa* on olive landscape services based on the general illustration of Emerton and Howard 2008.

non-market public good, are commonly examined by economists through non-market evaluation techniques (Figure 2) which are classified in two groups: revealed preference methods (i.e. market price, cost-based, hedonic pricing and travel cost) and stated preference methods (i.e. contingent valuation method, CVM and choice experiment, CE). The first group is limited to market goods and services, but based on observed user behavior. The second group can be applied to all goods and services to capture all use and non-use values, but based on hypothetical situations of users and non-users (Plan Bleu 2016). Among these techniques, CE is preferred for rural landscape evaluation (Scarpa and Cicia 2000; Scarpa et al. 2007), and is used for the purpose of this study for the following reasons: (i) it is suitable for evaluating multi-attribute ecological goods (Arriaza-Balmón et al. 2006), (ii) it minimizes strategic bias across a set of choices (Bennett and Blamey 2001; Hanley et al. 2001), (iii) it captures total economic value (use and non-use values) of the ecosystem, and (iv) it allows consideration of public preferences in the context of environmental goods such as olive landscapes (Sardaro et al. 2016b). Unlike CE, where the choice set is composed of at least three options (status quo and two hypothetical alternatives), CVM is based on only two possibilities (status quo and one hypothetical alternative).



**Figure 2.** Summary of various techniques used to assess the economic, social and ecological economic impacts of invasive species. The diagram assembles two categories of methods (revealed preferences and stated preferences) where Choice Experiment belonging to the second category was considered in our study.

There have been several studies of valuation of environmental services and damages in the past decades. These include the valuation of a damaged ecological public good through CVM as a useful approach in public policy formulation (Portney 1994), environmental valuation through CE (Hanley et al. 2001; Scarpa et al. 2007; Campbell and Hynes 2011; Hasund et al. 2011), assessment of environmental damage in monetary terms through the WTP (Johansson 1990), valuation approaches for natural ecological functions (de Groot et al. 2002), the elicitation of factors affecting citizens' behavior towards the prevention of environmental damage (Torgler and Garcia-Valiňas 2007), the estimation of WTP for rural landscape changes (Campbell 2007), individuals' preferences for agri-environmental services (Garrod et al. 2014), and economic quantitative valuation of damages due to climate change (Auffhammer 2018). At the Italian level, one specific study has quantified the economic and landscape impact of Xf in the Salento area (Sardaro et al. 2015). However, this previous research is based on a direct income loss approach, rather than on stated preference methods. The addedvalue of the present research is twofold. First, the monetary values of non-market olive landscapes features, based on individuals' preferences, have never been yet assessed in the affected area. Second, the present paper enriches the scientific literature that uses
CE to capture society's perception of research and experimentation activities, considered as an attribute in this study, to improve the representation of olive landscapes. The results have consequences for policy with respect to the recovery Plan (see below) for the damaged olive landscape and for pursuing ongoing research activities related to *Xf*.

## Methods

## Restoration Plan of the affected area

The Italian Ministry of Agriculture (Ministero delle politiche agricole alimentari, forestali e del Turismo, hereafter Mipaaft) created an Action Plan (2020) to prevent dispersal of the bacterium and rebuild the landscape in infected areas. This plan defines all measures to be taken to counter the spread of Xf in line with the Implementing Decision (EU) 2015/789 and the Ministerial Decree of 13.02.2018 (and subsequent amendments) to relaunch the agricultural and agro-food sector of the areas affected by the bacterium in Apulia. The Plan includes a set of measures, in particular (i) restoration of the damaged landscape (i.e. removal of damaged plants, replanting and conversions via resistant olive cultivars, reconversion to other crops, preservation of monumental olive trees via grafting with resistant varieties, support for growers' incomes during the transition period to new plants, and financial support for plant nurseries to readjust their structure and facilitate transfer in disease-free areas), and (ii) pursuit of future Xf research and experimentation (i.e. genetic and epidemiological, vector control, innovation of large-scale monitoring techniques, improvement and development of diagnostic techniques and innovative tools for the surveillance and prevention in the free areas, and new treatments for the bacterium).

## Agricultural overview of the study area

This study focused on the southern area of Apulia region, south-eastern Italy. Apulia has a total surface area of 1,954,052 hectares, distributed between its five provinces: Foggia (36%), Barletta-Andria-Trani (8%), Bari (20%), Taranto (13%), Brindisi (10%) and Lecce (14%). According to the National Statistics Institute (Istituto Nazionale di Statistica 2019), a variety of different crop types are present in Apulia. The agricultural area is mainly under cereals (29%), followed by table and oil olives (27%), temporary forage crops (17%), and permanent grassland, pastures and meadows (15%). In addition, Apulia accounts for 33.81% of Italy's olive-growing area and 33.22% of the country's olive production. The region's olive landscape is characteristic: 79% of the regional olive area is covered by trees over 50 years old (Ciervo 2016). In terms of trees, Apulia has 60 million plants on 382,600 ha (Istituto Nazionale di Statistica 2019), with around 3 million centenarian and millenarian plants, which capture particular cultural and aesthetic values (longevity from Roman times and the 17<sup>th</sup> century) on 90,000 ha because of their impressive trunks and contorted shapes. Nevertheless, the

Territory	Total area (1000 ha)	Table and oil olives area	Harvested production	Damaged area due to Xf	
		(1000 ha)	(1000 Tons)	(1000 ha)*	
Foggia	701	55	81		
Bari	386	100	298		
Taranto	247	35	69	4	
Brindisi	186	64	12	10	
Lecce	280	97	155	40	
Barletta-Andria-Trani	154	33	95		
Total (Apulia region)	1954	383	818	54	
Total (Italy)	30134	1166	2461	54	

Table 1. Total area, areas and harvested production of table and oil olives in 2019.

Source: Own elaboration based on data by www.istat.it. Extraction date: 19/04/2020. \* Italia Olivicola 2019.



Figure 3. Distribution of the area (in ha) damaged by Xylella fastidiosa in Apulia region.

olive area that has been completely lost due to Xf represents 14.06% of Apulia's olivegrowing area and 4.61% of the national total (Table 1; Figure 3). Moreover, Apulia's lost production for three years (2016–2018) is estimated at 29,000 tons (equivalent to €390 million), representing 10% of Italian olive production (Italia Olivicola 2019).

## Respondents' choice preferences: conceptual framework

In recent years, CE has been widely used to assess the non-market services of public goods (Dallimer et al. 2015; Zoderer et al. 2015; Sardaro et al. 2016b; Bottero et al. 2017; Tempesta and Vecchiato 2017; Cortignani et al. 2018; Novikova et al. 2019), and in particular to reveal respondents' (i.e. citizens, residents or tourists) preferences regarding the benefits of ecosystem services (Dachary-Bernard and Rivaud 2013). Based on utility theory (Lancaster 1966 as cited by Tempesta 2014), it has been assumed that respondents' utility for the olive landscape restoration is a function of

changes in landscape, additional costs faced to implement landscape restoration, and other control factors (i.e. research and experimentation, communication plan). Conditional logit models to explain respondents' preferences for olive landscape restoration have been estimated. A baseline model (model 1) disentangles the preference for a change in landscape. Further models investigate whether respondents' preferences differ according to geographical location and options for land restoration: conditional logistic regressions include interaction variables between locations (i.e. respondent resident in Foggia and Lecce) and changes in landscape (model 2) and between options for land (i.e. traditional, intensive, disperse/sparse, productive crops, productive crops with bushes) and changes in landscape (model 3). This investigation derives respondents' willingness to pay (WTP) as the ratio between the estimated parameter for options of landscape restoration and the negative of the parameter estimated for additional costs: each ratio reflects the average contribution a respondent would pay for landscape restoration. The rest of this section explores how these points have been addressed in this research. The options for landscape restoration have been defined on the basis of information retrieved from: the restoration Plan (Mipaaft 2020) and a focus group discussion. Landscape restoration presents 5 levels of changes (illustrated by corresponding photographs), listed in Table 2. As in previous studies on the quantification of Italian landscapes services (Tempesta and Vecchiato 2017), this research presents 5 levels of additional costs that citizens are willing to pay for landscape restoration  $(0, 15, 30, 60 \& 90 \notin \text{per household/year for the next 10 years})$ , illustrated by corresponding photographs. The experimental design includes also options for research and experimentation. The use of different photographs and/or pictograms was intended to help respondents in the choice process (Bateman et al. 2009; Zoderer et al. 2015). This visual information (Garrod et al. 2014) reflected the attributes under assessment to provide a stimulus to the respondent (De Ayala et al. 2012) and support the realism of the alternatives (Cherchi and Hensher 2015) in our CE experiment, in which respondents may easily select a choice set. The combination of the above attributes and their levels gave 120 possible scenarios ( $2^2 \times 5 \times 6$ ). A fractional factorial design (Christie et al. 2004; Bush 2013) was decided using the code of package AlgDesign on R, vielding 36 reasonable alternatives, which were divided into 3 blocks including 4 choices set each (see Table 3 as an example). Each choice set consisted of 4 columns. The first column described the areas of improvement (attribute) to guarantee the availability of the landscape for citizens today and for future generations. The next three

**Table 2.** Options for landscape restoration.

	Options for landscape restoration				
•	Status quo;				
•	Landscape with Xf-resistant olive cultivars only in a traditional production system;				
•	Landscape with Xf-resistant olive cultivars only in an intensive production system;				
•	Landscape with Xf-resistant olive cultivars only in a dispersed/sparse production system;				
•	Landscape planted with mixed productive crops (i.e. vineyards, pomegranate, stone fruits);				
•	Landscape planted with mixed productive crops and bushes.				

Area of improvement	Status quo	Alternative A	Alternative B	
Landscape	Landscape not restored, like now	Traditional olive production system	Landscape planted with mixed productive crops and bushes	
Research	Yes	No	Yes	
		(In the second sec		
Additional cost (10 years)	0 €/year	0 €/year	90 €/year	
Which option do you prefer?				
How certain are you about	1: □ Absolutely certain; 2: □ Quite certain;			
your choice?	3: □ Not very certain; 4: □ Completely uncertain			

Table 3. Example of a set choice.

columns concerned the three different scenarios (known as alternatives) that could be chosen. These had no labels but were referred to as: "*Status quo*", "*Alternative A*" and "*Alternative B*". For each respondent, 4 choice sets were presented in order to select the alternative that maximizes satisfaction requirements

Furthermore, the experimental design was organized into 3 blocks of 4 choice sets each, based on: (i) the previous experience of the focus group experts (Hoyos 2010) in Italian landscape valuation and conservation, (ii) the pilot survey, (iii) the prevention of complexity, minimization of confusion and cognitive fatigue for respondents during the survey, and (iv) the common use of manageable number choice sets (i.e. not higher than 5 to 6) in the CE literature (Caussade et al. 2005; Campbell 2007; Kallas et al. 2007; Garrod et al. 2014; Weller et al. 2014; Cherchi and Hensher 2015). The elicitation of respondents' preferences was examined through a social-choice sample survey in which the structure of the questionnaire had three sections. Section 1 included attitudinal questions on respondents' general attitudes towards the olive landscape and its benefits, as well as their knowledge of the multi-functionality of the olive sector in Apulia. At the end of this section, interviewees were informed about the current Olive Quick Decline Syndrome epidemic caused by Xf, which gradually deprives the Apulian landscape of its historical, patrimonial and cultural element: its olive trees. Two representative photos from the European Commission website showing the severity of the disease and the map of the demarcated area affected were shown to respondents in case any of them were unaware of the problem. Section 2 concerned their preferences for improvement of the affected landscape. In this section, 4 choice sets were presented to the respondents in order to select the option

that ensures the restoration of the affected landscapes in southern Apulia. In order to ascertain the certainty of their choices (Brouwer et al. 2010), participants were asked a question (how certain are you about your choice?) and indicated their answers on a scale from 1 (absolutely certain) to 4 (completely uncertain) at the end of each choice set. Furthermore, a set of statements was presented to respondents in order to best describe the way in which they made again their choices. Section 3 aimed to collect information about respondents' socio-economic characteristics (i.e. age, gender, residence, employment, sector of activity, membership of environmental associations, and annual income). An example of the questionnaire is enclosed in Suppl. material 1. A pilot survey was performed in order to define the time needed for face-to-face interviews and to guarantee that respondents fully understood the questionnaire. The final survey was carried out by qualified and trained agents in spring 2019, involving 683 respondents (general public) in three of Apulia's major cities (Foggia, Bari and Lecce), particularly in different contexts (i.e. in front of train and bus stations, public parks, local streets, etc.). Within each city, three blocks of the questionnaire were used. The Sections 1 and 3 of the questionnaire were kept constant, while Section 2 included four different sets of choices in each block. At least 60 people were interviewed randomly by block distributed between the sexes (at least 30 females and 30 males) and ages (at least 20 males and females for each of the following age ranges: 18 to 30, 30 to 50 and above 50 years old).

## Results

#### Basic descriptive statistics

#### Respondents' awareness and perception of Apulia's olive landscapes

This section includes basic statistical results from the Section 1 of the questionnaire (Suppl. material 1), dealing with respondents' general attitudes towards the olive landscape and its benefits, and their knowledge of the multi-functionality of the olive sector in the studied area. Observations of the natural landscape and historical buildings in Apulia were the most popular activities. The majority of respondents (45.1%) visited the Salento (damaged area) at some time within a year, while 33.1% of them live there, mainly in the Lecce area.

Although 17.1% of the participants in this social survey had not been there during the last 7 years, which means that they were not really aware of the incidence and severity levels of *Olive Quick Decline Syndrome* in the damaged area, almost all respondents (98.1%) recognized the relative importance of the presence of the olive trees in Apulia's landscape. With regard to their level of agreement about olive landscape services, very few respondents (0.6%) were convinced that this ecosystem does not provide benefits in Apulia, but 4.2% of them confirmed their ignorance about its ecosystem services. In general, the olive landscape obtained the highest agreement on its cultural heritage and aesthetic values (63.4% in terms of historical olive germplasm, great enrichment of the Apulia region, attractive appearance and evergreen), followed in succession by its other services: food production (59.7% for food security, olives & olive oils as common ingredients in the Italian diet, typical products), economic benefits (48.5%, including olive sales, tourism, gastronomy, hospitality, direct sale), positive environmental effects (46.9% related to biodiversity conservation, maintenance of native plants, animal life, sustainable agriculture, low use of chemical inputs, mitigation of the greenhouse effect, water management, prevention of soil erosion and run-off), social attributes (32.1% for the maintenance of family farming and rural employment), and finally, research features (27.7% as a field of research and experimentation).

#### Respondents' socio-economic profiles

According to latest data available on the website of Istituto Nazionale di Statistica (https://www.istat.it), our sample results (Table 4) are in a similar range to the main statistics of Apulia population (48.65% males; average age 44.7; average family size 2.5; annual average household income in the south of Italy  $\in$  32,807). On average, respondents were middle-aged (41 years old), and equally divided between genders since 51% were male, but were widely differentiated with respect to family size (1 member: 4.7%, 2 members: 16.8%, 3 members: 31.6%, 4 members: 38.9%, more than 4 members: 7.9%). The average family size was approximately 3.3. The majority (70.1%)

Variable	Categories	Median	Mean	Std. Dev.	Min	Max
Age	Year	39	41	16	18	86
Male	Male	1	0.51	0.50	0	1
	Female					
Resident in rural areas	Rural area	0	0.08	0.27	0	1
	Urban area					
Family members (Total number)	1	3	3.29	0.99	1	5
	2					
	3					
	4					
	5 & more					
Family members (Under 18 years old)	Number	0	0.40	0.70	0	4
Education level	1: Not educated	4	4.22	0.77	1	5
	2: Elementary school					
	3: Lower secondary school					
	4: High secondary school					
	5: University					
In work	Yes	1	0.67	0.47	0	1
	No					
Members of environmental associations	Yes	0	0.11	0.31	0	1
	No					
Olive grove owner	Yes;	0	0.32	0.47	0	1
	No					
Income level (€1000)	1: < 20	2	2	1	1	3
	2: between 20 mila and 60					
	3: > 60					

Table 4. The descriptive statistics of main variables for the entire sample.

of families had members over 18 years old. In terms of education, two major groups were observed: one with a high school diploma (about 38%) and one with a bachelor's degree (50.2%). The average educational level was about 4.22. The absolute majorities (66.6%) were mainly employees (38.5%), and total annual household revenue was distributed as follows: 25.5% (under  $\in 20,000$ ), 59.7% ( $\notin 20,000 - \notin 60,000$ ), and 14.8% (over  $\notin 60,000$ ). Most respondents (89%) were not members of an environmental association, while around 32% were owners of an olive farm with an average size of 0.75 ha. The overall descriptive analysis revealed a wide range of variables related to Sections 1 (opinion and awareness of the olive landscape in Apulia) and 3 (socio-economic profile) of the questionnaire.

#### Estimates of willingness to pay

Conditional logit models are estimated in order to disentangle potential heterogeneity in individual preferences. The results (Table 5, column 1) show that, as expected, additional costs required for landscape restoration are negatively correlated with respondent choices. With respect to changes in landscape, the correspondent coefficient estimate is positive, suggesting that local residents do not appreciate the "*status quo*" and tend to prefer other alternatives for landscape restoration. The average respondent is willing to pay  $\in$ 3.52 more for changes in landscape (for instance, to move from the *status quo* to a revived landscape). Considering the Apulian households (1,618,809 households), the average value that Apulians are willing to pay for landscape restora-

Variables	Baseline (1)	Location (2)	Type of landscape (3)
Cost	-0.008***	-0.009***	-0.016***
	(0.001)	(0.001)	(0.001)
Landscape	0.029***		
	(0.007)		
Landscape (Foggia)		0.049***	
		(0.010)	
Landscape (Lecce)		0.051***	
		(0.010)	
Landscape (Xf-resistant olive cultivars only traditional)			-0.707***
			(0.038)
Landscape (Xf-resistant olive cultivars only intensive)			-2.010***
			(0.077)
Landscape (Xf-resistant olive cultivars only dispersed/sparse)			1.775***
			(0.171)
Landscape (mixed productive crops)			0.496***
			(0.042)
Landscape (mixed productive crops with bushes)			0.173***
			(0.020)
Respondents	683	683	683
Observations	8,196	8,196	8,196
Pseudo R <sup>2</sup>	0.023	0.028	0.208

Table 5. Conditional Logit Models.

Notes: Standard errors are in brackets. P value:  $* = P \le 0.05$ ;  $**= P \le 0.01$ ;  $*** = P \le 0.001$ . All specifications control for 'research' and 'picture'. Bari is the baseline location in specification (2). *Status quo* is the baseline in specification (3).



**Figure 4.** Willingness to pay (WTP) for replacing the status quo with different types of landscape. WTP (in €) are obtained from (statistically significant) coefficients estimated in the conditional logistic regressions (Table 5, column 3). Lower and upper levels are in red colour.

tion is about 5.7 million of € per year. The preferences of respondents for changes in landscape tend to be homogeneous across locations (Table 5, column 2), but differ according to options for landscape restoration (Table 5, column 3). The estimated mean and 95% confidence intervals of WTP for options of landscape restoration are reported in Figure 4. Respondents are willing to pay €113.92 on average for Xf-resistant olive cultivars, suggesting public preferences for a sparsely covered olive landscape for ecological reasons (lower consumption of soil, low use of chemical inputs, and mitigation of the greenhouse effect), and increasing this kind of extensive land mainly for its cultural and aesthetic values. In addition, mixed productive crops and mixed productive crops with alternating bushes alternatives, are also preferred by respondents: the average WTP is €31.82 and €11.09, respectively. This indicates that local people tend to enhance the diversity of the rural landscape and biodiversity conservation of the Apulia region. However, the reconversion to other crops will be possible in conditions of profitability and economic sustainability (El Chami et al. 2020). Differently, respondents tend to not prefer landscape characterized by Xf-resistant olive cultivars only with a traditional or an intensive system of production. This implies that they do not have strong views about the traditional dense olive groves and their economic effects (direct selling, niche market), social provisions (maintenance of family farming, rural employment), job opportunities for unemployed persons as well as on food security, in which olives and oil are common ingredients in Apulia region. Assuming that the total estimated damaged area is 53,800 ha (Italia Olivicola 2019), and that the total number of households in Apulia is 1,618,809 (Istituto Nazionale di Statistica 2019), the mean value of loss of the socio-ecological benefits is 1,059 €/ha. The total number of families

was used instead of the total population, as stated by del Saz-Salazar and Menéndez (2007). However, the use of the total population will increase the mean value of loss to  $\notin 2,636$  per degenerated hectare.

#### Discussion

The findings explored in the Results provide a clear picture of respondents' opinions on the olive landscape, their preferences for the improvement of affected orchards, and their socio-economic profiles. Here we connect the observed results to the existing literature and derive some policy reflections from our findings. Firstly, our analysis highlights an extremely high level of perception of the cultural heritage and aesthetic benefits of the olive landscape. Previous research papers also assumed the importance of the assessment of cultural heritage values (Tengberg et al. 2012), as a subcategory of cultural ecosystem services (Hølleland et al. 2017) and aesthetic perception (Tribot et al. 2018) in landscape evaluation for sustainable land use planning and ecological restoration management. Rodríguez-Entrena et al. (2017) proposed the use of olive orchards to improve the aesthetic and visual quality of rural landscape's green cover. Secondly, our analysis shows that Apulia's inhabitants are willing on average to pay for alternative features in order to restore and revive the region's olive orchards. Obviously, the majority of respondents were found more likely to choose "landscape changes" over the current situation of degenerated land with diseased olive trees. Using plants and trees is hence preferred by citizens to mitigate the deterioration of rural landscape (Frontuto et al. 2020). Four types of alternative landscape features were proposed to the local public in the study area.

The highest WTP for landscape change was found for the landscape with Xf-resistant olive cultivars in a dispersed or sparse production system, followed by mixed productive crops, and then by mixed productive crops with Mediterranean bushes. Given this, the present study underlines the relative public preferences to crop diversity in landscape configuration heterogeneity as outlined by Hass et al. (2018) and sustains existent evidence to biodiversity conservation and ecosystem features. However, the substitution of olive orchards with native plants or Mediterranean bushes alone certainly requires less maintenance throughout the year, provides a wildlife habitat (plant reproduction, bee abundance) and allows landscape conservation (Slattery et al. 2003).

Meanwhile, this type of landscape gives the lowest direct incomes for local farmers. These types of landscapes were selected over a monoculture cropping system planted with Xf-resistant olive cultivars in a traditional production system. On the other hand, the landscape with resistant-Xf olive cultivars in an intensive production system was relatively the least attractive option for land restoration. The lack of preferences for this kind of landscape seems to decrease local public utility. This perception is in agreement with a recent study (Arata et al. 2020), in which the mean WTP of Italian inhabitants in Lombardy region (northern Italy), is only  $\in 6.7$  to increase the green area by 7%. Local citizens attach a specific reconversion to other crops which were already in the

past but appear to be gradually replaced by oil olive monoculture. These preferences diversify Apulia's agricultural landscape and enhance biodiversity in this region.

However, this kind of reconversion would be possible in conditions of profitability and economic sustainability over the new productions. Nevertheless, our findings support the results of Howley (2011), who found that intensive farming landscapes were less attractive for respondents. However, the dispersed production system provides fewer economic and social benefits than intensive production, as noted by Sardaro et al. (2016a). Furthermore, despite the fewer studies addressed to olive landscape valuation, the results of this research are relatively representative. A previous study by Marangon et al. (2008) assessed WTP for the introduction of olive trees into the landscape. The contingent valuation method applied on a hill region between Italy and Slovenia showed an average WTP of €25.59 household/year. Rodrígues-Entrena et al. (2017) stated that visitors' WTP per year is around €6.52 in terms of landscape restoration with parking fees to improve the aesthetic quality of the land. Torres-Miralles et al. (2017) estimated the WTP to sustain olive-growing with a natural reserve in Andalusia (Spain). Using CVM, the mean WTP was €37 household/year. Sardaro et al. (2016a) evaluated the benefits provided by olive landraces in Apulia, revealing that WTP for landscape preservation was €207 per year/family.

Furthermore, a number of studies elicited individual preferences in relation to environmental issues (such as landscape preferences) and multi-functionality of agriculture (Abler 2004; Dachary-Bernard and Rivaud 2013). Tempesta and Vecchiato (2017) have already reviewed previous Italian studies for evaluating landscape benefits. They found that WTP ranged from €2.8 to €74.3 per household/year. Ciaian and Paloma (2011) found that the mean WTP was €149 per ha, by using a metaanalysis technique of agriculture landscape valuation in EU. Our results highlight the relation between the mean WTP values and the distance from the degenerated landscape. Lecce residents live closer to the studied area and are relatively willing to pay more than Foggia residents (Figure 1). This proximity issue was analyzed by del Saz-Salazar and Menéndez (2007), who found a positive correlation between WTP and residence proximity. This issue was also stressed by Arata et al. (2020) as needing to be explored in future studies. The third contribution from the present study is the use of research as a conceptual contribution in designing choice experiment. Studies using this type of attribute are absent from the literature review for evaluation of ecosystem services.

#### Conclusion

Environmental issues such as restoration of damaged landscape are of crucial importance for land use development plans at regional, national and European levels (European Landscape Convention 2000). This convention urges each EU Member State "to assess the landscapes thus identified, taking into account the particular values assigned to them by the interested parties and the population concerned". In consequence, by assessing WTP and preferences of the local public towards alternative features or changes in landscape through the stated preferences approach, our study constitutes an efficient prerequisite tool to support local policy-makers on the allocation of financial resources in the best interest of the local inhabitants in the study area. This evidence was recently noted by Rewitzer et al. 2017; Arata et al. 2020 and Frontuto et al. 2020. Assessment of the social value of the damaged olive landscape in Puglia is based on landscape changes and pursuit of the ongoing research and experimentation on Xf. The latter attribute is a specific challenge for the Italian landscape, given the economic impact of Xf subsp. pauca on olives. Reinforcement of the ongoing research on the production of transgenic olive plants and on vector control (i.e. tillage, weed management, use of insecticides, trapping, monitoring and surveillance) is necessary to reduce the economic impact of Xf. Schneider et al. (2020) have highlighted the importance of the strengthening research to reduce the future spread rate of Xf from 5.18 to 1.1 km/ year on Italian, Spanish and Greek olives. In fact, the production of resistant cultivars of olives is an important axis of research to cope with Xf invasion. In addition, through the replantation of potential damaged landscape by using resistant olive cultivars, the hypothetical economic impact of Xf on olives in these countries can also be reduced from a range of €3.58 to 8.69 billion (without replanting resistant olive cultivars) to a range of  $\in 2$  to 4.13 billion (by replanting resistant cultivars), over a period of 50 years. Thus, the present study has important policy implications for the current regional recovery action plan (Mipaaft 2020) in the study area, particularly for the restoration and revival of damaged olive orchards, control of Xf vectors and enhancement of research and experimentation aimed at finding a cure for this disease. Lastly, the present choice experiment model could be enhanced by involving other landscape attributes related to the level or types of crops for reconversion, the safeguarding of ancient/monumental olive trees, and communication activities. This research could also be extended to cover the preferences of tourists and of local entrepreneurs for whom improvement of the olive groves would have positive impacts on income.

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# Supplementary material I

## The questionnaire (1 block of 3 is presented hereafter)

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DISCUSSION PAPER



# Alleles and algorithms: The role of genetic analyses and remote sensing technology in an ant eradication program

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#### Abstract

Eradication programs for invasive ants are often hampered by a lack of effective tools to detect, contain and kill the pests. Among the range of tools employed in the course of a 20-year eradication program for red imported fire ant, Solenopsis invicta, in Australia, two of the most crucial for success are genetic analysis at both individual colony and population scales, and remote sensing for the detection of S. invicta mounds over large areas. Several genetic analyses are used by the program as an everyday operational tool to guide the eradication effort; for example, genotyping of the social form determines where and how far we need to search and treat, whereas nest relatedness derived from microsatellites aids in deciding when and where to target investigations into human-assisted movement of the pest. Microsatellite genotyping can determine the origin of new invasions into the country and has been used to verify the eradication of six distinct incursions of S. invicta in Australia, as well as demonstrating the pressure being exerted on the remaining Queensland population by the current eradication activities. Remote sensing played a key role in delimiting the extent of the S. invicta infestation in southeast Queensland in 2015, and in the future will assist in both delimitation and in verifying eradication of this ant in treatment areas as part of the proof of freedom process. Unquestionably, without these tools, the battle to eradicate S. invicta from Australia would be severely constrained, if not lost. These technologies may be applicable in management or eradication programs for S. invicta worldwide, and potentially for other invasive ant species.

#### **Keywords**

Invasive ants, red imported fire ant, Solenopsis invicta

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## Introduction

The red imported fire ant, Solenopsis invicta Buren is a notorious invasive ant species which has become established in several countries around the world, causing economic and ecological damage and affecting health and lifestyle (Wetterer 2013). In Australia, a national program (the program) for the eradication of S. invicta, has been in operation since 2001 when the ant was first discovered at two locations in Brisbane, Queensland (Vanderwoude et al. 2003). Responses to these and subsequent incursions in other parts of Australia are part of the overarching national program. The methodology employed in this program and some of its successes have been outlined by Wylie et al. (2016). Six of the nine known incursions of S. invicta into Australia have been eradicated (five in Queensland and one in New South Wales), including the world's largest ant eradication which was one of the original infestations at the Port of Brisbane (8300 hectares) (Hoffmann et al. 2016; Wylie and McNaught 2019). Efforts continue against the remaining southeast Queensland infestation, which now covers an area of over 500 000 hectares. Recently, at the end of 2019, an incursion of S. invicta was detected at the Port of Fremantle in Western Australia from China, and another at the Port of Brisbane from the United States in early 2021. Localised eradication responses have commenced for both incursions.

While eradication of *S. invicta* from Australia has not yet been achieved, and aside from the new incursions at the ports of Fremantle and Brisbane, a measure of the efficacy of the program is that the remaining southeast Queensland infestation has been successfully restricted to a relatively small area of the State, with no known spread to other parts of the country from this population. In 2015, the extent of this infestation was delimited by Keith et al. (2019) with a 99.9% level of confidence, and over the life of the program its rate of spread has averaged 4.8 km per year (Wylie et al. 2019). In comparison, the rate of spread of *S. invicta* in Texas over a 10-year period was 48 km per year (Hung and Vinson 1978) and 80 km per year in China (Lu et al. 2008). The slow rate of spread in Australia has been attributed to the program's surveillance and treatment regimes, community support, and strict controls on the movement of products likely to harbour *S. invicta* (Wylie et al. 2019).

These achievements have been substantiated through the use of two of the program's most valuable tools against the pest – genetic analyses to determine origin of new invasions, social form and population genetic structure, and remote sensing for detection of *S. invicta* mounds. The manner in which these tools are employed to inform and guide program strategy and management decisions is described in this paper, along with a case study that demonstrates the benefits of genetic analyses.

#### **Genetic analysis**

#### Social form

*Solenopsis invicta* colonies contain either a single queen (monogyne) or multiple queens (polygyne), whereas many other of the world's invasive ant species are solely polygynous

(Tsutsui and Suarez 2003). In its native range, *S. invicta* colonies are mostly monogyne, with occasional polygyne colonies that typically contain several closely related queens (Ross et al. 1996). In the United States, it wasn't until the 1970s that polygyne colonies, often containing many unrelated queens, were officially recognised (Ross et al. 1996). Early speculation on polygyny was that it developed in introduced populations of *S. invicta* in response to ecological constraints (e.g., Keller 1995). More recent studies suggest a separate introduction of the polygyne form into North America subsequent to the establishment of monogyne *S. invicta* (Tsutsui and Suarez 2003; Shoemaker et al. 2006).

The social form of a colony/nest is determined in a laboratory by genotyping of the Gp9 locus from DNA extracted from 10 whole worker ants (pooled) from the same sample and subjected to High Resolution Melt (HRM) Polymerase Chain Reaction (PCR) (Oakey et al. 2011). This discerns the presence of the Gp9 alleles B and b. Where all workers are found to be homozygous BB it is concluded that the colony is monogynous, whereas the presence of the b allele indicates the colony is polygynous. In Brisbane, genotyping revealed that both social forms of the ant were introduced in 2001 at the Port of Brisbane and at Richlands in the west of the city (Wylie et al. 2016).

The reproductive forms display different physical, behavioural, reproductive, and genetic traits. For instance, monogyne colonies spread predominantly by flight while polygyne colonies primarily spread by budding off a new colony overground (King et al. 2009) or by movement of infested materials. As such, determining social form of populations and individual colonies assists in assessing the level of spread risk posed by infestations and facilitates appropriate eradication activities. The program adapts its response depending on the social form; for example, surveillance buffers for a monogyne detection (with strong-flying founding queens) are greater than for a polygyne detection (that predominantly spread only short distances by budding or flight). However, polygyne colonies are of particular concern for several reasons, both in terms of impact to their surroundings and spread risk. Specifically, the higher nest densities in polygynes pose a greater risk to human and animal health, economic impact and the environment. As polygynes have multiple queens - nearly 700 have been recorded in a single mound in the United States (Glancey et al. 1975) - there is a higher likelihood of spread by human assistance (King et al. 2009) in soil or other carriers and by flooding (Biosecurity Queensland, unpublished data) than for monogynes. Additionally, due to the nest density and hierarchical feeding strategy (Tschinkel 2006) they take longer to kill (e.g., an average 4-5 rounds of insect growth regulator bait treatments compared to 3-4 rounds for monogynes (Biosecurity Queensland, unpublished data)).

For the reasons outlined, elimination of polygynes from an *S. invicta* population is an important contributor towards its successful eradication. Monitoring by the program of the distribution and the frequency of polygyne colonies in southeast Queensland shows that there has been a significant reduction in the proportion of polygynes in the population from almost 40% in 2001 to approximately 1% in 2018–2019 (Fig. 1). This reduction has been attributed to targeting of polygyne infestations by the program through chemical treatment and controls on product movement. In comparison, in countries without effective controls or eradication programs for *S. invicta*,



**Figure 1.** Proportion of sites with the polygyne social form of *S. invicta* in the Brisbane populations. Brisbane data are from the period 2001 to 2019, with the minimum and maximum of this social form in Taiwan and USA populations also portrayed.

the incidence of polygyny is 24–92% in Taiwan (Yang et al. 2008), 15–54% in the United States (Porter et al. 1991; Porter 1992) and in China the majority of colonies are polygyne (Yang et al. 2012).

Occasionally, the determination of a colony as polygyne is unexpected, either because they have not been found in an area before, are found in isolation, or are detected in an area where monogyne colonies predominate. From the perspective of determining the illegal movement of fire ant carriers or tracing the source of the infestation, it is of benefit to determine whether these 'nests of interest' could be a result of occasional, long-distance flight events of polygynes or the more likely movement of material containing queens or alates. In such cases, 10 individual workers are genotyped with HRM PCR. If the workers of a polygyne nest are determined to have a ratio of Bb:BB:bb alleles represented by approximately 2:1:1 (or at least to show a mixture), then this is indicative of an established polygyne colony that is either a) likely to have other undiscovered nest mounds in the same area, or b) from a rebuilt nest following assisted movement of one or more queens. Both of these situations require further responsive actions from the program beyond elimination of that nest. However, if all workers are determined to have a *Bb* genotype, it is possible that the nest was established by an occasional surviving BB alate (mated winged queen) from a polygyne colony, referred to as a 'heavy queen' because of her higher body weight compared to a *Bb* polygyne alate.

Most heavy queens are executed by the colony prior to flight and therefore seldom survive to undertake a nuptial flight (see Tschinkel 2006). Alternatively, the nest may have been founded by a monogyne queen that mated with a polygyne b male. In southeast Queensland, the majority (approximately 80%) of these 'nests of interest' are considered movement of fragments from established nests with the remaining 20% being a nest founded through a heavy queen or monogyne queen mating with a b male. As those colonies genotyped are a subset of the total number of polygyne colonies detected, it is concluded that nest founding by heavy queens occurs at only a low frequency. The presence of nests with this Gp9 genotypic composition (i.e., all workers with a Bb genotype) highlights the potential risk of mating events between polygynes and monogynes as a source of genetic variation and the associated risk to the current genetic bottleneck within the southeast Queensland population (discussed further below).

#### Population structure

Genetic analysis of population structure can provide information on changes in the genetic diversity of populations of the ant. A reduction in genetic variation and the occurrence of inbreeding or population fragmentation may result in reduced fitness and adaptability of the population and indicate program success. A single ant worker from each monogyne colony sampled is genotyped at thirty-seven microsatellite loci. These loci were selected from the 72 used in the global study by Ascunce et al. (2011) as those that show allelic diversity in the Queensland populations. At the time of writing, this database consists of over 36000 colony genotypes. Fragment analyses are performed with Applied Biosystems 3500xL genetic analyser. Microsatellite alleles are determined and proofread using commercial software Genemarker (Soft Genetics via Millennium Science, VIC.) (Ascunce et al. 2011).

Every 1–2 years, the accumulated and annual genotype data are analysed with Bayesian clustering algorithms via the software Structure (Pritchard et al. 2000) to establish the number and spatial distribution of genotypic clusters. A cluster is defined as a collection of genotypes that are more similar to each other than to genotypes outside the cluster. The number of clusters is tested using two methods, (Pritchard et al. 2000 and Evanno et al. 2005). Once the number of clusters is identified, multiple simulations are run with a high number of iterations to assign a probability of each sample belonging to each cluster. Simulations are averaged across runs to a single assignment probability table using CLUMPP software (Jakobsson and Rosenberg 2007).

Long-term analyses of the clusters have shed light on the change in structure of the population over time, and the success of the program. The proportion of the total genetic variance contained in a subpopulation relative to the total genetic variance ( $F_{ST}$ , calculated by GenAlEx; Peakall and Smouse 2006) showed that in the first five to six years following discovery of the ant in Queensland (2001–2006), the main Brisbane population consisted of a single genetic cluster ('Main'), the Port of Brisbane population having been eradicated pre-2005–06 (Wylie et al. 2016). In 2006–07 a subcluster emerged in the west of the infestation ('West') and in 2009–10 another sub-



**Figure 2.** Schematic summarizing the temporal fluctuations of sub-clusters of the Brisbane population of *S. invicta* over the period 2001 to 2019.

cluster emerged in the east ('East'). A second western sub-cluster ('West 2') emerged in 2010–11 from the Main cluster, not from the West sub-cluster. Similarly, a second eastern sub-cluster ('East 2') emerged in 2011–2012 from the Main cluster (Fig. 2).

The formation of these sub-clusters may be indicative of the pressure being exerted on the remaining Brisbane population by the program's eradication efforts. The expectation for a *S. invicta* incursion after 20 years was that there would be no decrease in genetic variation and limited sub-structuring of the population due to genetic mixing via natural mating, migration, and human-assisted transport. The opposite of this is occurring in Queensland where there is lower genetic diversity than is reported in other invaded countries, evidence of inbreeding and population fragmentation. This has not been observed in *S. invicta* populations in countries without effective control or eradication programs (Ascunce et al. 2011). In 2018–2019, the East sub-cluster was no longer distinguishable from the Main which may indicate a weakening of the genetic bottleneck (see below) in that sub-cluster.

#### Population assignment

When *S. invicta* is detected well outside known areas of infestation, an immediate concern is whether this is spread from an existing population or a new incursion. To address this critical question, there is an extensive global database on the genetics of the ant from which comparisons can be made, again using microsatellite markers (Ascunce et al. 2011). A sample of 10 individuals is genotyped using the microsatellites as above. The alleles are compared to the global data and potential sources are eliminated

if the test sample contains alleles that are not associated with that population. Paetkaus assignment tests in GenAlEx (Peakall and Smouse 2006) are applied to consider the likelihoods of assignment to the different sources.

Using this global database, the assignment tests have enabled the program to pinpoint the origin of all incursions to date. The two 2001 Brisbane incursions, and incursions at the Port of Gladstone in 2013, Brisbane Airport in 2015, and Port of Brisbane in 2021, were from the southern United States. The incursions at Yarwun in 2006, Port Botany in 2014 and Port of Brisbane in 2016 were from Argentina, and the 2019 Fremantle incursion was from China. None of the incursions post-2001 were related to the original Brisbane populations or to each other. Such information allows the program to prove that the subsequent incursions were not as a result of spread from southeast Queensland or that the Port of Gladstone infestation was not the result of eradication failure at the nearby earlier Yarwun incursion. It also shows that the program generally has been effective in containing spread of populations to other parts of Australia.

#### Relatedness

Determining relationships, particularly parentage, between individual *S. invicta* colonies can provide a range of information that is used by the program for strategic or operational purposes.

Examples of the type of information derived include: (a) providing data on the distances flown by newly mated monogyne *S. invicta* queens for input to models used to predict spread; (b) providing spatial information on the direction and rate of spread of monogynes to guide treatment and surveillance activities; (c) assisting in identifying potential cases of non-compliance with movement restrictions; (d) differentiating between reinfestation of a previously infested area and persistence of a colony following pesticide treatment of that area.

Relatedness estimates are derived from the R-coefficient in haplodiploid (see Bourke and Franks 2019) models. The genotype of the monogyne queen is determined from 10 individual workers and analysed using the software Kingroup (Konovalov et al. 2004), along with manual confirmation through comparison of alleles (see Case Study below).

#### Genetic bottleneck

When a population undergoes a reduction in its numbers, there is typically a reduction in genetic variation through a loss of rare alleles which can be revealed by microsatellite genotyping of loci that are not under selection (i.e. neutral with respect to selection). When a low number of individuals from a stable population establish in a new area (as with incursions of invasive pests such as *S. invicta*), this new population undergoes an extreme form of bottleneck, referred to as the 'founder effect' (Mayr 1954; Tsutsui and Suarez 2003). A reduction in genetic diversity generally is associated with negative effects that put strain upon the survival of the reduced population. Examples include reduced ability to adapt or evolve to new situations, reduced capacity to resist parasitoids or disease, and a necessity to breed with kin thus exacerbating these effects (Garza and Williamson 2001; Schmid-Hempel et al. 2007). Therefore, establishing the bottleneck of the *S. invicta* populations and sub-populations on an annual basis provides information relating to the effective pressure on, or recovery of, 'genetic fitness'.

Detection of a bottleneck is made through comparing the expected heterozygosity (see Gregorius 1978) with the observed heterozygosity, and the expectation that populations in genetic bottleneck would show an average heterozygosity excess when compared with the source population or population in genetic equilibrium (Maruyama and Fuerst 1985). On the other hand, a heterozygosity deficit may indicate signs of establishment or recovery. Levels of heterozygosity are determined annually by applying the software Bottleneck (Cornuet and Luikart 1996) to datasets from each of the clusters defined by Structure. Results of analysis in 2019 show that all remaining clusters within the Brisbane population remain in strong bottleneck. All clusters are showing increasing or maintained pressure over time and allele frequencies show evidence of inbreeding, which implies increased stress and reduction in fitness (Garza and Williamson 2001). These analyses also revealed that the Port of Brisbane incursion that was discovered in 2001, pre-dated the Richlands incursion discovered in the same year. Specifically, the early Port of Brisbane data show a recovering population post-establishment, which later demonstrated extreme fitness stress (Biosecurity Queensland, unpublished data) following extensive, repeated treatment that resulted in eradication of this population (Wylie et al. 2016).

#### Case study: Port of Gladstone 2013

An example of how the program uses genetic analysis to effectively respond to a significant new discovery of S. invicta is the 2013 incursion at the Port of Gladstone in central Queensland. Genetic analyses of samples collected at the port facility and nearby industrial sites during the investigative stage of the response showed that all samples were monogyne and the origin was determined as the southern United States. The incursion was unrelated to either of the Brisbane populations or to the 2006 incursion at nearby Yarwun (approximately 4 km away), which originated from Argentina. The knowledge that the colonies were monogyne helped to determine the extent of the surveillance zone. Research in the United States showed that 99% of newly mated queens of monogyne S. invicta flew less than 1.6 km unaided by wind (Markin et al. 1971) and an energetics study by Vogt et al. (2000) estimated their flight capability, and ability to successfully found a nest, at <5 km in the absence of wind. Surveillance at the Port of Gladstone was therefore conducted out to a 6 km radius from confirmed infestation to delimit the extent of the incursion. More than 6000 hectares of suitable habitat were surveyed by field teams and fire ant odour detection dogs, and the size of the infested area was determined to be 220 hectares (Wylie et al. 2016).

In the three months following initial detection, samples from 66 colonies were analysed. A pedigree or family tree of the Port of Gladstone incursion was constructed using a combination of approaches. R co-efficients were used to infer *S. invicta* relationships from Kingroup (Konovalov et al. 2004) and to manually add to and adjust the pedigree to best fit the data. Manual application allowed for flexibility and possibilities such as inbreeding to be accounted for. Bottleneck (Cornuet and Luikart 1996) analysis was conducted to determine heterozygosity excess or deficit. From these analyses it was concluded that:

a) the incursion was likely founded by two queens;

b) the population was in a distinct genetic bottleneck, with a strong bias towards heterozygosity excess associated with a very recent founding event;

c) the incursion had been present for approximately six generations, which establishes its minimum age at around three years, about the time this section of the Port was constructed;

d) fourteen colonies needed to complete the pedigree tree were not sampled, five of which are at the top of the tree and may not have survived. Therefore, it is estimated that only nine colonies were not identified through the surveillance activities, which equates to an efficacy rate of 88%. Four of those colonies were subsequently detected, which increases the efficacy rate of combined human and odour detection dog surveillance at the site to 93%;

e) inbreeding/breeding with kin was notably high, particularly during the first generations;

f) the pedigree tree depicts near-exponential amplification of the infestation;

g) the mean post-mating flight distance for the mainland infestation was 420 m, ranging from 29 m to 1.2 km.

There were two long-distance movements to nearby Curtis Island of 3.8 km and 4.6 km, but it is not known if this was flight or via human assistance. The majority of flights (70%) were from the west to the east, which is against the prevailing onshore wind (from the east for 11 months of the year according to local meteorological data). This differs from the results of a United States study where Rhoades and Davis (1967) found 90% of newly founded colonies downwind from their source population. It is generally agreed that *S. invicta* favour highly disturbed habitats (see Tschinkel 2006) and this is also supported by unpublished program data from Brisbane where approximately 70% of all finds are associated with major soil disturbance, predominantly residential, commercial and road development. At the Port of Gladstone, the presence of a large expanse of suitable habitat at the site (specifically, bare ground) may have had a greater influence on the establishment of newly mated queens than wind direction (Fig. 3).

We found evidence that the Gladstone infestation experienced pleiometrosis (collaborative founding of nests by multiple monogyne queens) which can result in relatively higher fitness of colonies. Pleiometrosis has been reported previously in the United States (Tschinkel and Howard 1983; Bernasconi and Strassman 1999; Manfredini et al. 2013) and can result in higher survival and growth rates in the claustral period (when the first generation of workers is raised) than for nests founded by a



**Figure 3.** Photograph of Fisherman's Landing, Port of Gladstone in central Queensland. The large expanse of bare ground was attractive habitat for newly mated *S. invicta* queens, with the majority of nests found on this site along the edges of drains where moisture was present or in isolated grass clumps.

single queen. Ultimately, only one queen will survive and gain reproductive dominance. We concluded pleiometrosis was likely because, unexpectedly for monogyne colonies, over 20% of the nest samples were found to consist of workers from more than one queen and over half of these had 2–4 families present. In these cases, the dominant genotype was accepted as the nest identity, and the other workers labelled as 'tourists'. While one explanation for the 'tourists' is poor sampling technique resulting in mixed samples, this is unlikely given that the operations teams were all experienced and such a result from the same teams had not occurred previously. Another possibility is that samples contained foragers from surrounding nests as well as in-nest workers. Monogyne colonies are generally aggressive to workers from other colonies, although the high level of inbreeding and genetic inter-relatedness of nests may confuse the recognition of self/kin and territorialism that would normally combat or repel the 'tourists'.

Pleiometrosis is more common where mated queens are in high density or where suitable habitat is scarce. This is consistent with the main site of infestation at the Port of Gladstone, which was challenging for colony founding, being reclaimed sand and coral fragments and a 'hard stand' of compressed crusher dust and gravel. The majority of the nests found in those areas were along the edges of drains where moisture was present or in isolated grass clumps. This potential pleiometrosis at the Port of Gladstone may be related to 'microtopography' (limited suitable habitat available in the landscape resulting in clumping of founding queens (Tschinkel and Howard 1983). As most 'tourists' could not be assigned to a source nest, it is postulated that they may be residual workers from pleiometrotic founding events.

#### Remote sensing surveillance

#### Background

The detection and delimitation of the extent of the infestation is one of the major challenges with invasive ant incursions. Indeed, it has been purported that an inability to detect all nests will either expand the time and cost of eradication, or inevitably lead to failure to eradicate (Hoffmann et al. 2010). In the early years of the program, surveillance was conducted solely by on-ground field staff, either visually or using lured pitfall traps or 'hot dog' sausage lures. When there was visible mound building or soil disturbance by the ants (usually in the cooler months or after heavy rain), visual surveillance provided detection rates of around 80% based on quality assurance evaluations. However, human visual surveillance is expensive and slow, as are pitfall traps and lures. Early in the program, as infestations began to be detected in less-populated peri-urban and rural areas, it became apparent that a more efficient method was needed for large-area surveys. A range of methods was investigated, with aerial remote sensing selected as the most suitable option for broad scale surveillance. Initial trials conducted in late October 2004 using infrared and colour video cameras to detect S. invicta mounds were unsuccessful. This was attributed to technical limitations for image capture, data storage and processing, and the timing of flights in warm weather when there was little temperature differential between the background and the target (Biosecurity Queensland, unpublished data). Subsequent advances in technology combined with successful trials of remote sensing for S. invicta in the United States (Vogt 2004a, b; Vogt et al. 2008a, b) led to the program's reinvestigation into the use of remote sensing in mid-2008.

Impetus to move remote sensing surveillance out of research and development and into operation came following an independent review of the program conducted in 2009–2010. The review concluded that eradication of the remaining *S. invicta* population in southeast Queensland was not feasible using existing techniques and recommended that research on remote sensing surveillance be completed within two years. Remote sensing surveillance became operational in 2012 with the primary aim of delimiting the southeast Queensland infestation.

#### Early remote sensing surveillance 2012–2015

Early research and development into remote sensing in the program realised three main technological components in the remote sensing surveillance process: capturing aerial imagery, analysing the imagery to identify potential *S. invicta* mounds,

and follow-up field surveillance to investigate those potential mounds. The camera system used by the program during this period was developed collaboratively by an Australian company (Outline Global Pty. Ltd.) in partnership with United States companies that had approved access to patented technology under the control of the US military. The system consisted of a camera pod containing six discrete high-resolution cameras; three visible spectrum (red, green, blue), one near-infrared and two long-wave thermal infrared. The cameras were chosen to maximise the chance of detecting *S. invicta* mounds through analysis of size, shape, colour, texture, vegetation cover and heat. The camera pod was mounted to the undercarriage of a helicopter that flew at a height of approximately 400 feet above ground level at a minimum speed of 30 knots.

#### Image capture

Remote sensing image capture was conducted in the cooler months of the year (May to September in Brisbane) when S. invicta mound temperatures can be considerably warmer than the surrounding ground. For example, in Brisbane, differences of up to 20-30 °C (average +11.9 °C, n = 1467) have been recorded, making them highly visible with thermal imagery, whereas recorded temperature differences were only up to 10 °C between mounds and their surroundings in Mississippi studies (Vogt et al. 2008a). As reported from remote sensing trials in the United States, a range of factors can affect the capture of suitable imagery, including nest location (obscured or in the open), mound shape, weather conditions, time of year and soil type (Vogt 2004a, b; Vogt et al. 2008a, b; Vogt et al. 2009). Similar findings were made in southeast Queensland and incorporated into operational guidelines. For example, one of the main factors affecting the thermal signature of a mound is cloud cover. Field observations in 2012 demonstrated that mounds cool rapidly when the sun is blocked by cloud, and within half an hour can be the same temperature as the surrounding ground. From this, an Aerial Operations Weather Guide and Ratings System was developed to help guide decisions on the suitability of conditions for flying. Flying is only conducted at Rating 1 (no visible clouds in the target area or the surrounding areas) or Rating 2 (high cloud and less than 10% of the sky with clouds). Mound temperature monitoring also provided information on the optimal times of day to fly; no earlier than 9 am and no later than 3.30 pm, except on the longer days in early spring. However, around the time of the winter solar azimuth when the sun is at its lowest angle, flights did not commence until 10 am and finished at 3 pm. It was also determined that mounds could be detected on the day following heavy rain provided that the mound was exposed to the sun. However, mounds in long grass were not detected unless the sun was overhead, and mounds at the bases of trees or in tree shade were difficult to detect. The shape of the mound was also important - dome-shaped mounds with a height of more than 15 cm provided the best thermal signature, while mounds that had been flattened and disturbed (e.g., by mowing) were generally undetectable.

Windy conditions may cause aircraft vibrations that resulted in image distortion, and steep terrain occasionally made it difficult for the pilot to maintain a consistent speed and height above the ground.

## Image processing algorithm

Imagery was first georeferenced to establish the image location in respect to map projections and coordinate systems, and orthorectified to remove the effects of image perspective (tilt) and relief (terrain). The processed imagery was then analysed using a customdesigned machine-learning algorithm. One of the limitations in the development of the algorithm used in the 2012–2015 period was a lack of training images. At that time, focus was given to killing colonies as quickly as possible rather than retaining suitable sites for image capture. Although the algorithm had high detection rates when settings were adjusted to ensure no false negatives, it produced too many false positive points of interest for staff to follow up with field surveillance. Consequently, a manual analysis process was introduced whereby each point of interest identified by the algorithm was then assessed by a trained technician who would recommend to either discard or follow-up on a point. Using these two systems, and with further training of the algorithm, points of interest were reduced to operationally acceptable levels of about two per hectare.

## Field surveillance

Point of interest surveillance involved field staff navigating to defined coordinates identified by the algorithm and manual analysis process, and then conducting a search in a 10 m radius around that point to confirm whether or not an *S. invicta* colony was present. If a mound was detected then an additional 500 m of field surveillance was conducted to detect any additional mounds in the area, this being the distance limit for 90% of *S. invicta* alates as determined by program genetics (see case study above) and work in the United States (Tschinkel 2006). Surveillance was extended if new mounds were found until there were no further detections.

## Remote sensing results

Over the period 2012–2015, a total of 218 000 hectares of remote sensing surveillance was completed on the fringes of the known infested area to delimit the infestation at that time. When a new detection was confirmed, then the next round of surveillance was pushed out to 5 km beyond that detection, as this is the estimated distance limit for a newly mated queen to fly and successfully establish a colony (Vogt et al. 2000). Data obtained from remote sensing and from other program surveillance activities conducted during that period were analysed by a spread model developed by Keith et al. (2019) who concluded that the southeast Queensland infestation had been delimited with a 99.9% level of confidence as of May 2015.



**Figure 4.** A comparison of the sensors investigated for use in detecting fire ants by remote sensing. Imagery was captured from a helicopter at 700 ft, with the five sensors including (from left to right): ultraviolet, very near infrared, short wavelength infrared, medium wavelength infrared and long wavelength infrared. A confirmed fire ant nest is present around a rock in the middle of images (white circle in right image) and is particularly apparent as bright yellow in both medium and long wavelength infrared. Imagery gathered in collaboration with Outline Global Pty Ltd, Australia.

#### Future remote sensing surveillance

The 2012–2015 delimitation of the infestation in southeast Queensland was a key factor that led an independent review of the program in 2015–2016 to conclude that eradication of the pest was still technically feasible and in the national interest. The review panel recommended the continuation of the eradication program and the development of a new response plan (Magee et al. 2016). Remote sensing surveillance ceased in June 2015 because the cameras were near or past the maximum shutter actuations recommended, and in need of refurbishment or replacement. Acquisition of next-generation remote sensing technology was necessarily delayed until funding was secured for the new response program that commenced in July 2017. Research and development on improved systems for image capture (including additional bandwidths; Fig. 4) and analysis (artificial intelligence deep-learning algorithm), data storage and field surveillance is nearing completion and the first operational flights are planned for 2021.

## Discussion

As outlined by Shoemaker et al. (2006) there have been numerous studies on the genetic structure of ants at various scales to assess, for example, relatedness within and between social groups and the nature of queen-worker conflict, or to determine patterns of dispersal and gene flow and reveal relationships between native and introduced populations of invasive species. In Australia's eradication program for *S. invicta*, genetics is used as an everyday operational tool to guide the immediate eradication effort. For example, knowledge of the social form of a new detection determines the extent of the search area around that detection, what treatment will be applied to the site and, if polygyne, whether investigations into human-assisted movement are warranted. Similarly, circumstances of a colony discovery may raise questions of non-compliance of movement restrictions of material containing monogyne alates, and the microsatellite genotype database is applied in an attempt to identify possible sources for investigation. Genetics is also used strategically to guide longer term planning and activities. Evidence of inbreeding and fragmentation in Brisbane *S. invicta* populations, implying reduced genetic fitness as a result of program activities, validates the program's efforts and bolsters the confidence of stakeholders that eradication is still achievable (see Magee et al. 2016).

Remote sensing surveillance similarly exploits aspects of the biology of *S. invicta*. The heat signatures of *S. invicta* mounds in the cooler months of the year can be captured in thermal imagery and their habit of keeping their mounds clear of vegetation is an additional marker that can be discerned by near infrared imagery as it produces a 'halo' effect around the mound. With the assistance of artificial intelligence algorithms, *S. invicta* mounds can be distinguished from those produced by other ant species such as meat ants (*Iridomyrmex purpureus*) or by mound-building termites in southeast Queensland (mainly grass-eating termites *Nasutitermes* species). The latest sensors and algorithm can also confidently distinguish *S. invicta* mounds from rocks and manufactured objects. Remote sensing surveillance has a key role to play in the ongoing program both for delineating the extent of the infestation and also assisting in verifying eradication of *S. invicta* in treatment areas as part of the proof of freedom process.

Whilst remote sensing has been used in pest management to detect changes in the environment caused by insects and fungal pathogens e.g., changes in plant health based on canopy spectral signatures (Stone and Mohammed 2017; Prasannakumar et al. 2020) and has been used widely to detect invasive plants (Bradley 2014; Niphadkar and Nagendra 2016), its use in invasive ant establishment is in its infancy. Considering the potential capability of remote sensing to detect fire ant nests over large areas (tens of thousands of hectares), this would provide a more efficient method compared to current ground surveillance activities.

## Conclusion

Our program is the first time genetics and remote sensing surveillance have been used as routine, operational tools in an ant eradication program and particularly at the scale employed in southeast Queensland. Unquestionably, without these tools, the conclusions of the 2015–2016 independent review would have been very different, and the focus of the program would then have switched from eradication to managing and slowing the spread of the pest as has been necessary in other countries. The approaches we have used increase the chances of eradication of the red imported fire ant, thus avoiding the considerable economic and environmental impacts of this invader, which would be to Australia's detriment. These methods and frameworks could be applied to management and eradication efforts for *S. invicta* worldwide. Potentially, these technologies could be adapted for use against other invasive ant species.

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RESEARCH ARTICLE



# Food-web modification in the eastern Gulf of Finland after invasion of *Marenzelleria arctia* (Spionidae, Polychaeta)

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### Abstract

The paucity of data on non-indigenous marine species is a particular challenge for understanding the ecology of invasions and prioritising conservation and research efforts in marine ecosystems. Marenzelleria spp. are amongst the most successful non-native benthic species in the Baltic Sea during recent decades. We used stable isotope analysis (SIA) to test the hypothesis that the dominance of polychaete worm Marenzelleria arctia in the zoobenthos of the Neva Estuary after its invasion in the late 2000s is related to the position of this species in the benthic food webs. The trend towards a gradual decrease in the biomass of Marenzelleria worms was observed during 2014–2020, probably due to significant negative relationships between the biomass of oligochaetes and polychaetes, both of which, according to SIA, primarily use allochthonous organic carbon for their production. The biomass of benthic crustaceans practically did not change and remained very low. The SIA showed that, in contrast to the native crustacean Monoporeia affinis, polychates are practically not consumed either by the main invertebrate predator Saduria entomon, which preys on *M. affinis*, oligochaetes and larvae of chironomids or by benthivorous fish that prefer native benthic crustaceans. A hypothetical model for the position and functional role of *M. arctia* in the bottom food web is presented and discussed. According the model, the invasion of *M. arctia* has created an offshoot food chain in the Estuary food webs. The former dominant food webs, associated with native crustaceans, are now poorly developed. The lack of top-down control obviously contributes to the significant development of the Marenzelleria food chain, which, unlike native food chains, does not provide energy transfer from autochthonous and allochthonous organic matter to the upper trophic levels. The study showed that an alien species, without displacing native species, can significantly change the structure of food webs, creating blind offshoots of the food chain.

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#### **Keywords**

NIS, stable isotopes, bioturbation, detritivores, macroinvertebrates, zoobenthos, Neva Estuary

# Introduction

Biological invasions are widely recognised as a key component of current global change. Human livelihoods and well-being in almost all regions of the world depend on alien taxa (Kumschick et al. 2020). Recently, the effects of invasive species may be exacerbated due to climate change (Moritz and Agudo 2013; Holopainen et. al. 2016; Golubkov 2021). For example, the expected rise in water temperature in the northern part of the Baltic Sea may lead to frequent invasions and spread of the more thermophilic Ponto-Caspian species in this region (Holopainen et. al. 2016). In addition, ever-increasing shipping raises the probability of introduction of alien species from any region. The paucity of data on non-indigenous marine species (NIS) is a particular challenge for understanding the ecology of invasions and prioritising conservation and research efforts in marine ecosystems (Ojaveer et al. 2015). Some NIS may have economic consequences on fisheries and the tourism industry and can result in substantial monetary costs and/or alterations to entire ecosystems and social systems (Kumschick et al. 2012, 2015; Galil et al. 2014; Ojaveer et al. 2015). Therefore, monitoring for the presence of impacting alien species is a requirement for the management of marine environment (Lehtiniemi et al. 2015).

Amongst the other approaches, food webs, which describe the trophic links amongst species in a system, are particularly important for studying the impacts of invasions (David et al. 2017). For instance, invasion can trigger a trophic cascade, affecting the abundance of the main trophic groups in the food web (Penk et al. 2015). Moreover, the impact is enhanced when the invader is an ecosystem engineer, belongs to a new functional group or affects the abundance of a keystone species (David et al. 2017). The effects of marine invaders also depend on trophic position and functional similarity. For example, invaders, across studies, typically have negative effects on biodiversity within a trophic level, but positive effects on biodiversity of higher trophic levels (Thomsen et al. 2014). They also can directly or indirectly alter (a)biotic characteristics of ecosystems, resulting in changing energy flows through the food web.

*Marenzelleria* spp. belong to the 68 most widespread NIS in European Seas (Galil et al. 2014; Cardeccia et al. 2018). In the Baltic Sea, these polychaete worms are amongst the most successful non-native benthic species during recent decades. They were first found there in 1985 and quickly spread throughout the Sea, becoming the dominant species in the zoobenthos of many areas (Blank et al. 2008; Kauppi et al. 2015; Maximov et al. 2015). Studies carried out by both morphological and molecular methods have determined that these polychaetes belong to three related species, *M. viridis* (Verrill), *M. neglecta* Sikorski and Bick and *M. arctia* (Chamberlin) (Sikorski and Bick 2004; Bastrop and Blank 2006; Blank et al. 2008). The first two species came from North America (Sikorski and Bick 2004). The latter species was previously found only in the Arctic (Jørgensen et al. 1999; Sikorski and Bick 2004). At present, *M. arctia* is prevalent in the deep regions of the northern Baltic Sea (Kauppi et al. 2015; Maximov 2015).

Marenzelleria worms function as ecosystem engineers by modifying the physical, chemical and biological characteristics of bottom sediments (Kristensen et al. 2011; Kauppi et al. 2015; Maximov et al. 2015). The native benthic macrofauna of the northern Baltic Sea is low in diversity and being strongly dominated by a few key species: the bivalve Macoma balthica and the crustaceans Monoporeia affinis (Lindstrom), Pontoporeia femorata Krøyer and Saduria entomon (Linnaeus) (Bonsdorff 2006). Indigenous polychaete worms in the northern part of the Baltic Sea are generally few in number and there are no deep-burrowing species amongst them. Invasive polychaetes burrow deeper than local zoobenthos species, causing bioturbation and bioirrigation of bottom sediments. Biological irrigation by Marenzelleria spp. promotes deeper penetration of oxygen into sediments and retention of phosphorus in them (Maximov et al. 2015). This, in turn, caused a decrease in the concentration of phosphates in water in some ecosystems of the northern Baltic and contributed to the improvement of the state of the environment by preventing eutrophication and harmful cyanobacterial blooms. However, the effect of oxygenation of bottom sediments becomes significant only at high densities of Marenzelleria worms (Berezina et al. 2019). Hence, it is important to forecast changes in the abundance of this species.

It has long been observed that some invaders decline after a period of extremely high abundance (Simberloff and Gibbons 2004). This usually occurs through long-term restructuring of food webs and communities after an invasion and increasing top-down or bottom-up control of invaders (David et al. 2017). Therefore, determining the place that a new invader occupies in the food webs of the recipient's ecosystem is very important. The explosive development of non-indigenous worms *Marenzelleria* occurred in the eastern part of the Gulf of Finland in 2009 after a series of hypoxic – anoxic events that led to the decline of native zoobenthos communities, which were dominated by the amphipod *M. affinis* and the isopod *S. entomon*, over a large area of the Gulf (Maximov 2015). Currently, *M. arctia* dominates the zoobenthos of the easternmost Gulf of Finland (Maximov 2015; Golubkov et al. 2017, 2019).

Stable isotope analysis (SIA) provide new possibilities to investigate aquatic invasion risks and their associated impacts, as it can be an important tool to elucidate the trophic structure and carbon sources in food webs (Middelburg 2014). SIA gives longterm and time-mediated information on consumed trophic resources and is used to describe quantitatively the trophic relationships occurring amongst organisms. SIA can detect potential diet overlap and feeding competition between species and estimating the proportion of different preys and carbon sources in the diet (e.g. Zwerschke et al. 2018; Golubkov et al. 2019; Verstijnen et al. 2019; Golubkov et al. 2020a; Haubrock et al. 2020). Accordingly, it can be used to investigate and predict the impact of invasive species on native ones and on the whole local communities (Haubrock et al. 2020). The aim of the study was based on 7-year observations to determine the modern trophic position of *M. arctia* in the benthic food webs of the eastern Gulf of Finland in order to assess the prospects for the development of this species in the area. We tested the hypothesis that the dominance and high role of these polychaetes in the benthic macroinvertebrate communities of the Estuary is related to the position of this species in benthic food webs, in which this species is not affected by predators. This goal was attained by performing stable isotope analysis (SIA) of the tissues of zoobenthos and fish. We applied Bayesian mixing model (SIAR; Parnell et al. 2010) to quantify basal resources of various consumers. Outputs from the Bayesian models are in the form of the probability distributions, rather than just summaries of all feasible solutions. As a result of the informative predictions generated by the Bayesian mixing model approach, the SIAR modelling method has become a standard quantitative application for estimating diet resources (Layman et al. 2012; Colborne et al. 2016; Golubkov et al. 2018, 2019).

### Materials and methods

### Study site and sampling

The Neva Estuary, which is located at the top of the Gulf of Finland (Figure 1), receives water from the Neva River, the most full-flowing river of the Baltic Region, whose flow averages 2492 m<sup>3</sup> s<sup>-1</sup> (78.6 km<sup>3</sup> year<sup>-1</sup>). It is brackish-water, non-tidal, shallow, with horizontal and vertical gradients of salinity. The morphometric and hydrochemical features of the Neva Estuary have been previously described in many publications (e.g. Golubkov and Alimov 2010; Golubkov and Golubkov 2020).

At present, heavy nutrient and organic matter loading, mainly from the Neva River and St. Petersburg City (the largest megalopolis in the Baltic Region with > 5 million citizens), are the most significant environmental problem for the Neva Estuary. Eutrophication, organic pollution and biological invasions are the most serious threats to the environment of the Neva Estuary (Telesh et al. 2008; Golubkov et al. 2017, 2018, 2020b). NIS have a significant impact on the Estuary environment, many of which have become the dominant species in macroinvertebrate and fish communities over the past two decades (Orlova et al. 2004; Golubkov and Litvinchuk 2015; Maximov 2015; Demchuk et al. 2021).

Samples were collected in the middle part of the Neva Estuary (Figure 1). The salinity of waters near the surface in this part of the Estuary ranges from 1 to 3 PSU and of near bottom waters up to 5 PSU; the depth ranges from 7 m at sampling station 7 and up to 24.5 m at station 5. Zoobenthos was sampled at seven stations (Figure 1) with a modified Van Veen grab ( $20 \times 20$  cm) in early August 2014–2020. Samples were sieved in a 0.25 mm mesh with filtered fresh water and preserved in 4% formaldehyde in plastic zip-bags. Large crustacean *S. entomon* (Isopoda) were collected by trawling. Benthic animals for SIA were taken to the laboratory alive. Fish were collected with hook and line and frozen in the laboratory.



**Figure 1.** The upper and middle parts of the Neva Estuary with indication of sampling stations (1–7). Black lines: isobaths of 5, 10 and 20 m. Areas with dots indicate dense reeds. C1, C2 – gates for vessels; D1–D6 – waters gates in the St. Petersburg Flood Protection Facility. Anchor marked passenger and cargo ports. Red rectangles – the location of the Neva Estuary. Two-letter country codes are given according to ISO 3166-1 alpha-2 (ISO 2021).

# Sample analysis

Invertebrates were picked out of the samples under a stereomicroscope, identified, counted and weighed to the nearest 0.1 mg. Abundance and biomass of animals (wet weight, shells of molluscs included) were estimated as an arithmetic mean  $\pm$  SEM (standard error of the mean) from seven replicates and re-calculated per 1 m<sup>2</sup> of bottom area.

Live animals for SIA were identified under stereomicroscope and separated by species into vessels containing filtered water. Animals were maintained alive during 2 days to allow gut clearance. Faecal material was removed periodically to prevent coprophagy. After 2 days, animals were dried at 60 °C for 48 h. Small conspecific individuals were homogenised in an agate mortar to make a composite sample. Samples consisting of muscle tissue were used in the case of large animals (*S. entomon* and fish) as was recommended by Keough et al. (1996). Homogenised animal tissue from composite samples were put into small tin capsules and weighed using a Mettler Toledo MX 5 balance with an accuracy of  $\pm 1 \mu g$ . At least three replicates of each type of organism or material were prepared and analysed.

The SIA was performed according to standard methods (Keough et al. 1996) using a Thermo Delta V Plus isotope mass spectrometer (Thermo Scientific, United States) equipped with an element analyser at the Joint Usage Center "Instrumental Methods in Ecology" of the A.N. Severtsov Institute of Ecology and Evolution of RAS (Moscow, Russian Federation). Isotopic composition of C and N in organic matter was expressed in  $\delta$ -notation relative to the international standard (vPDB for carbon and the atmospheric N<sub>2</sub> for nitrogen):  $\delta$  (‰) = (R<sub>sample</sub>/R<sub>standard</sub> – 1) × 1000, where R = <sup>13</sup>C/<sup>12</sup>C or <sup>15</sup>N/<sup>14</sup>N. Samples were analysed with reference gas calibrated against IAEA (Vienna, Austria) reference materials USGS 40 and USGS 41. The drift was corrected using an internal laboratory standard (casein). The standard deviation of  $\delta$ <sup>13</sup>C and  $\delta$ <sup>15</sup>N values in the laboratory standard (n = 8) was < 0.2‰. We did not conduct a lipid extraction from animal tissues because it may bias the estimation of trophic links of consumers (Tarroux et al. 2010).

# Data modelling

The SIAR v.4.2 package (Parnell and Jackson 2021) running in R Software (version 3.5.1; R Development Core Team 2021; www.r-project.org/) was used for estimating trophic links of the consumers, based on their  $\delta^{13}$ C and  $\delta^{15}$ N values. SIAR (Stable Isotope Analysis in R) is a mixing model, based on hierarchical Bayesian methods, used to estimate contributions of basal sources, taking into account uncertainty and variability of input data, as well as isotopic enrichment factors (Parnell et al. 2013).

Estimates of the diet of individual organisms were obtained for two-isotope models using the 'SIARsolo' command. The mixing models were run using iterations – 500,000, burn-in – 50,000 and thinning by 15, without using concentration dependencies. Model solutions were presented using credibility intervals (95%, 75%, 25% and 5%) of probability density function distributions (Parnell et al. 2010). To compare the proportions of each source for a group on the boxplots 'siarproportionbygroupplot' command was used. The sensitivity analysis was carried out on fractionation factors as recommended by Inger et al. (2006). Values for trophic fractionation of C and N isotopes were chosen from the C and N range, given in Michener and Kaufman (2007). When an acceptable solution could be found, the simulation results varied by a maximum of 5% for the average contribution to the diet. An acceptable solution was found with trophic enrichment ratios of 1.63‰ for  $\delta^{13}$ C and 3.56‰ for  $\delta^{15}$ N from Parnell et al. (2013).

# Results

# Composition and biomass of zoobenthos

Alien polychaete *M. arctia* dominated in the study area during the period of research (Figure 2). *Potamothrix hammoniensis* (Michaelsen) and *Limnodrilus hoffmeisteri* Claparède (Oligochaeta), as well as larvae of *Chironomus plumosus* (Linnaeus) (Diptera, Chironomidae), were subdominant. Indigenous crustacean *S. entomon* (Isopoda) and *M. affinis* (Amphipoda) that dominated in zoobenthos before 2000s (Maximov 2003; Golubkov and Alimov 2010) were rare.

The biomass of polychaete worms significantly decreased in 2014–2020 (Figure 3A). On the contrary, the biomass of oligochaetes showed a positive tendency during this period (Figure 3B). The biomasses of other groups, as well as the total biomass of zoobenthos, did not show any tendencies. Significant negative relationships (R = -0.44, p = 0.002, n = 46) were observed between the biomass of oligochaetes and polychaetes (Figure 4).

# Stable isotope analysis and SIAR results

The isotopic signatures of key benthic macroinvertebrates and fish species varied in a wide range, reflecting differences in the use of different resources and in the trophic level (Figure 5).

According to the SIAR modelling, the previously highly dominant invertebrate predator in natural communities, the isopod *S. entomon*, mainly fed on amphipod *M. affinis* and the larvae of *Ch. plumosus* (Figure 6A). *M. affinis* has also been an impor-



**Figure 2.** Mean biomass (g WW/m<sup>2</sup>)  $\pm$  the standard error of the mean (SEM) of the dominant zoobenthic groups and their portions (%) in the total biomass of zoobenthos in 2014–2020.



**Figure 3.** Changes in the biomasses of *Marenzelleria arctia* (**A**) and oligochaetes (**B**) in 2014–2020. Vertical bars are  $\pm$  SEM.



**Figure 4.** Relationships between the biomass of oligochaetes and polychaetes in the Neva Estuary in 2014–2020.



**Figure 5.** Isotopic signatures ( $\delta^{13}$ C and  $\delta^{15}$ N values, mean  $\pm$  SEM) of common zoobenthic species and fish in the Neva Estuary. Cp – *Chironomus plumosus*, Lh – *Limnodrilus hoffmeisteri*, Ph – *Potamothrix hammoniensis*, Mo – *Monoporeia affinis*, Mar – *Marenzelleria arctia*, Se – *Saduria entomon*, Ab – *Abramis brama*, Rr – *Rutilus rutilus*.



**Figure 6.** The proportion of the use of various prey by the predatory macroinvertebrate *Saduria entomon* (**A**) and the fish *Rutilus rutilus* (**B**) and *Abramis brama* (**C**) in the Neva Estuary according to the SIAR model. The dark grey, grey, light grey and white are 95%, 75%, 55% and 5% credibility intervals. The numbers indicate the average percentages in the diet for 95% probability. Cp – *Chironomus plumosus*, Lh – *Limnodrilus hoffmeisteri*, Ph – *Potamothrix hammoniensis*, Mo – *Monoporeia affinis*, Mar – *Marenzelleria arctia*, Se – *Saduria entomon*.

tant resource for roach and bream (Figure 6B, C). In turn, *S. entomon*, played a significant role in the feeding of bream (Figure 6C). All of these predators poorly consumed alien polychaetes *M. arctia*, which dominated amongst non-predatory invertebrates.

In accordance with the data on the biomass of consumers and SIA, polychaete worms form the dominant food chain in the food web in the middle part of the Estuary (Figure 7), which apparently plays an important role in energy flow and functioning of the benthic macroinvertebrate community. This food chain is actually a closed branch of the Estuary food webs, since *Marenzelleria* is hardly eaten by secondary consumers. The former dominant food webs, associated with native crustaceans, are now poorly developed.



**Figure 7.** Food web and the share of various carbon resources in the diet of the main consumers in the ecosystem of the Neva Estuary. The use of autochthonous and allochthonous carbon is given according to Golubkov et al. (2019). The thickness of the arrows is proportional to the biomass of consumers.



**Figure 8.** A hypothetical model for the position and functional role of *Marenzelleria arctia* in the bottom food web of the Neva Estuary. Arrows indicate directions of organic matter transfer.

# Discussion

# Composition and succession of zoobenthos

An important goal of invasion biology is to identify environmental characteristics that may make a region particularly receptive to invasions (David et al. 2017). While some ecologists consider abiotic conditions that are favourable for survival of exotic species (Moyle and Light 1996; Lahdes and Karjala 2007), others focus on their biotic interactions of invaders with native biota (e.g. Ptáčníková et al. 2015; Vanderploeg et al. 2015). Moreover, some studies have shown that if abiotic factors are appropriate for an exotic species, this species is likely to invade successfully, regardless of the native species already present (Moyle and Light 1996; Marchetti et al. 2004; Golubkov et al. 2020b). However, low species and functional diversity also can open an ecosystem for alien species, because unsaturated niche space makes communities more vulnerable for invasions (David et al. 2017). The highest numbers of non-indigenous species were found in the transit water systems in the Baltic Sea: in the Gulf of Finland, the Szczecin Lagoon and the Vistula Lagoon (Zettler et al. 2014). These water systems have wide gradients of environmental characteristics and relatively low species diversity.

Salinity gradient and hypoxia events are the main driving forces of zoobenthic succession in the Baltic Sea area (Rumohr et al. 1996; Bonsdorff 2006; Zettler et al. 2014). Due to low salinity and periodic hypoxia stress, benthic communities in the northern Baltic Sea are species poor and are dominated by few species that currently affect ecosystem functioning (Villnäs et al. 2012). At the same time, freshwater and euryhaline species increase biodiversity in the freshened parts of the Baltic. Amongst them are species that benefit from organic and nutrient enrichment (Balushkina and Golubkov 2018; Golubkov et al. 2019).

All these trends are actual for the zoobenthic community in the middle part of the Neva Estuary. Historical data show that, at the beginning of the last century, benthic communities were rather species-poor and dominated by indigenous crustaceans, *Monoporeia affinis* and *Saduria entomon* (Golubkov and Alimov 2010). Later, due to the increase in organic pollution from St. Petersburg, the oligochaetes, *Potamothrix hammoniensis* and *Limnodrilus hoffmeisteri* and the larvae of *Chironomus plumosus*, which are typical in fresh or slightly brackish polluted waters, also became common. This community existed until the mid-1990s, when, after a series of hypoxic events, the biomass of zoobenthos, especially of hypoxic sensitive crustacean, as well as the energy flow in benthic communities, decreased many times (Golubkov and Alimov 2010; Golubkov et al. 2010; Maximov 2015). The restoration of benthic communities was associated with the development of the alien polychaete *Marenzelleria arctia*, firstly recorded in 2009, which became the dominant species of zoobenthos (Maximov 2011).

When introduced, a species may persist only if it is able to pass through environmental and biotic filters (David et al. 2017). Biotic filters include resource availability, competition with native species and predators. A decrease in the diversity and abundance of communities due to disturbances makes ecosystems less resistant to invasions, since it reduces competition with native species and frees up niche space (Shea and Chesson 2002). The current warming of the climate mainly contributes to the introduction of relatively thermophilic species from middle latitudes to more northern latitudes (e.g. Holopainen et al. 2016). However, in the case of *M. arctia*, the opposite direction of invasions was observed. This species is native to the coastal zone of the Arctic seas, where it is mainly found in river mouths (Sikorski and Buzhinskaya 1998; Sikorski and Bick 2004). It possibly entered the Baltic Sea from the White Sea with the ballast waters of ships through the White Sea-Baltic Canal, which includes a system of lakes and artificial canals (Figure 1). However, the construction of the canal was completed back in 1933. The naturalisation of *M. arctia* in the eastern Gulf of Finland only seven decades later was apparently facilitated by the temporary decline of native zoobenthos species due to hypoxic-anoxic events.

In subsequent years, in 2014–2020, the biomass of *M. arctia* decreased (Figure 3). However, the biomass of benthic crustaceans remained practically unchanged and remained very low. This means that, despite a significant decrease in the invader biomass, the community did not return to its original state and confirms the opinion that, even in those cases where there may be a reasonable expectation of decline, an introduced species can leave a trace long after its influence has weakened (Simberloff and Gibbons 2004). The reason for this may be associated with a change in the configuration of food chains after the introduction of an alien species into the native community.

### SIAR modelling and food webs structure

Changes in food web structure following invasion might, in most cases, be mainly related to changes in trophic group abundances rather than to species extinctions, as suggested by a meta-analysis on aquatic ecosystems (Gallardo et al. 2016). The invasion of alien *M. arctia* also did not lead to the extinction of native benthic species. Instead, according to SIA results, it formed its own food chain with low connectivity to the rest of the Estuary food web (Figure 7).

In the Neva Estuary, *M. arctia*, as well as dominant oligochaete and chironomid species, mostly used allochthonous wastewater-derived carbon as a basal resource for their production (Figure 7; Golubkov et al. 2019). Oligochaetes *L. hoffmeisteri* and *P. hammoniensis* (Oligochaeta) and larvae of *Chironomus* spp. are important indicators of polluted waters and benefit from organic pollution (Saether 1979; Rodriguez and Reynoldson 2011). They reach high abundance in the eutrophic and polluted waters of the Baltic Estuaries (Wolnomiejski and Witek 2013; Balushkina and Golubkov 2018; Kornijów et al. 2019; 2021). On the contrary, non-indigenous *M. affinis*, in addition to allochthonous carbon, largely uses autochthonous carbon produced by phytoplankton (Figure 7; Golubkov et al. 2019). This species consumes phytoplankton and phytodetritis, which is deposited on the seabed throughout the Baltic Sea (Lopez and Elmgren 1989; Lehtonen and Andersin 1998).

*M. arctia* consumes surface sediments and suspension around their burrows (Renz and Forster 2013). Therefore, polychaetes and the amphipods likely compete for food

resources, as both are deposit-feeding animals partly sharing similar food resources (Kotta and Ólafsson 2003). Interspecific and intraspecific competition for food was considered to be the main mechanism regulating the population size of *M. affinis* in the field (Wenngren and Ólafsson 2002; Kotta and Ólafsson 2003). Hence, it can be assumed that the development of the abundant polychaete population after the hypoxia events of the early 2000s prevents the recovery of the *Monoporeia* population. Moreover, an innate tendency of *M. affinis* to move up upon disturbance from burrowing activities of neighbours may result in enhanced susceptibility to predators (Lopez and Elmgren 1989). Thus, the successful competition of *Marenzelleria* with *Monoporeia* may be facilitated by the fact that the population of the latter, unlike polychaetes, is under the influence of *S. entomon* and fish (Figure 7).

It has often been observed that NIS populations decline after an initial period of high abundance (David et al. 2017). In the Neva Estuary, we also observed a progressive decline of *M. arctia* biomass during 2014–2020 (Figure 3A). Significant negative relationships were found between the biomass of oligochaetes and polychaetes in the Estuary (Figure 4). The reason may lie in interspecific competition for resources between polychaetes and oligochaetes, since both groups mainly use allochthonous carbon for their production (Figure 7; Golubkov et al. 2019). However, consuming organic matter from different layers of sediment can prevent food competition (Kornijów et al. 2021). L. hoffmeisteri and P. hammoniensis (Tubificinae, Oligochaeta) ingest food particles from deep sediment layers and defecate faeces at the sediment surface (Davis 1974; Rodriguez and Reynoldson 2011; Kornijów et al. 2021), while Marenzelleria spp. collect particles at the surface around burrows and defecate faeces deep into sediments (Renz and Forster 2013). Therefore, the activity of worms of these zoobenthic groups causes differently directed fluxes in bottom sediments. The bioturbation activity of polychaetes causes the transport of solid and dissolved organic matter from the surface to the deep layers of bottom sediments. They enrich the deep sediment layers with organic matter and stimulate microbial particles reworking in them (Renz and Forster 2013). On the contrary, the bioturbation activity of oligochaetes causes an upward transfer of old-age substances contained deep in bottom sediments, which reduces the content of organic matter on their surface (Davis 1974; Kornijów et al. 2021). Accordingly, this upward conveyor can negatively affect the feeding conditions of *M. arctia* if the oligochaete biomass increases. On the other hand, this can hardly lead to the complete elimination of Marenzelleria from the system, due to the sedimentation of phytoplankton and other organic particles from the water column. Most likely, this should help stabilise the biomass of polychaetes at some equilibrium level.

SIAR modelling showed that the main invertebrate predator, *S. entomon* and fishes do not feed on *M. arctia*. As a result, the food chain leading to polychaete worms practically does not interact with other food chains of the benthic food web (Figure 7). The probable reason is that *Marenzelleria* spp. burrow deep into bottom sediments (Renz and Forster 2013). Their burrows probably allow these species to effectively avoid predation by surface invertebrate predators and fish. Instead, surface-dwelling

amphipod *M. affinis* is a preferred food item for *Saduria* (Ejdung and Elmgren 2001). This crustacean is also a favourite food of fish in the Neva Estuary (Kudersky et al. 2007). Subsurface-dwelling larvae of *Ch. plumosus* and burrowing oligochaetes are less important sources of carbon for secondary consumers (Figures 6, 7). The vertical distribution of zoobenthos is an important trait of its suitability for benthic-feeding fish (e.g. Moss and Timms 1989; Persson and Svensson 2006; Weigel and Bonsdorff 2018). In an estuary of the southern Baltic, the Darss-Zingster Bodden Chain, the only recognisable remains of *M. viridis* in bream, carp and flounder were the bristles (Winkler and Debus 1996). These were usually taken in with detritus. Whole *Marenzelleria* in the guts of these fish were never found. However, they have been found in small numbers in the guts of pikeperch. It probably consumed polychaetes during their nocturnal migration into the water column (Winkler and Debus 1996).

A hypothetical model for the position and functional role of *M. arctia* in the bottom food web is given in Figure 8. The invasion of *M. arctia* has created a new offshoot in the estuarine food webs. The lack of the top-down control obviously contributes to significant development of this food chain. Unlike native food webs, this food chain does not provide energy transfer from autochthonous and allochthonous organic matter to the upper trophic levels and obviously negatively affects demersal fish production. This means that NIS can not only fill in the free nodes in food webs, but also reconstruct food chains, diverting resources that were previously consumed by other species and directing them along chains that are not always useful for humans. On the other hand, earlier there were no burrowing polychaetes in the Neva Estuary and the invasion of *Marenzelleria* formed a new functional group in its ecosystem (Maximov et al. 2015). Their activity leads to bioturbation and bioirrigation of bottom sediments and stimulates the decomposition of organic matter by worms and microorganisms (Figure 8). However, all these processes deserve further detailed research.

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RESEARCH ARTICLE



# A weed risk analytical screen to assist in the prioritisation of an invasive flora for containment

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### Abstract

Prioritising weeds for control and deciding upon the type of control and its associated investment are fundamental to weed management planning. Risk analysis is central to this process, combining the activities of risk assessment, risk management and risk communication. Risk assessment methodology has a rich history, but management feasibility has typically been a secondary matter, dealt with separately or not at all. Determinants of management feasibility for weeds include the stage of invasion, weed biology, means of control and cost of weed control. Here, we describe a simple weed risk analytical screen that combines risk assessment with species traits that influence management feasibility. We consider stage of invasion, species biological/dispersal characteristics and plant community invasibility in a preliminary analysis of the risk posed by the non-native plant species on Christmas Island in the Indian Ocean. For each of 31 high-risk species considered to be ineradicable under existing funding constraints, we analyse the risk posed to two major plant communities: evergreen closed-canopy rainforest and semi-deciduous scrub forest. Weed risk ratings are combined with ratings for species-intrinsic feasibility of containment (based on a measure that combines time to reproduction with potential for long distance dispersal) to create preliminary rankings for containment specific to each community. These rankings will provide a key input for a more thorough analysis of containment feasibility - one that considers spatial distributions/ landscape features, management aspects and the social environment. We propose a general non-symmetric relationship between weed risk and management feasibility, considering risk to be the dominant component of risk analysis. Therefore, in this analysis species are ranked according to their intrinsic containment feasibility within similar levels of risk to produce an initial prioritisation list for containment. Shadetolerant weeds are of particular concern for the closed-canopy evergreen rainforest on Christmas Island, but a greater diversity of weeds is likely to invade the semi-deciduous scrub forest because of higher light availability. Nevertheless, future invasion of both communities will likely be conditioned by disturbance, both natural and anthropogenic. The plant communities of Christmas Island have undergone significant

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fragmentation because of clearing for phosphate mining and other purposes. With a substantial number of invasive plant species firmly established and having the potential to spread further, minimising future anthropogenic disturbance is paramount to reducing community invasibility and therefore conserving the island's unique biodiversity.

### **Keywords**

Christmas Island, containment, dispersal, disturbance, eradication, invasibility, island ecosystems

# Introduction

Risk analysis comprises three activities: risk assessment, risk management and risk communication (MacDiarmid and Pharo 2003; Vanderhoeven et al. 2017). Risk assessment, the evaluation of the threat (hazard) posed by a potentially invasive species, in conjunction with the likelihood of the threat being realised, has been employed to prioritise the management of invasive non-native species but generally fails to consider management feasibility (Booy et al. 2017; Bartz and Kowarik 2019). Bartz and Kowarik (2019) have recently reviewed the state of play for weed risk assessment in the broader context of the assessment of environmental impacts for harmful non-native plant species (hereafter generally referred to as 'weeds'). They recommend that assessments should incorporate context dependence of environmental impacts, as well as the prospects for successful management.

Weed risk management provides a structured evaluation of management options. It has received relatively little attention to date (Booy et al. 2017; Vanderhoeven et al. 2017; see also Bertolino et al. 2020 and Kumschick et al. 2020 for broader considerations of invasive organism risk management), but there has been a move by invasion scientists and practitioners to develop scoring protocols to assess the susceptibility of species to various management options (Booy 2015, cited in Vanderhoeven et al. 2017; Wilson et al. 2017). Among other considerations, these protocols are based on information relating to species distributions and abundance, the likelihood of reinvasion, the effectiveness of management options, relevant legislation and public acceptance of the management measures (Vanderhoeven et al. 2017).

Eradication as a weed management goal has been rather comprehensively investigated following seminal publications (Rejmánek and Pitcairn 2002; Panetta and Timmins 2004; Cacho et al. 2006). Wilson et al. (2017) provide a recent summary on the topic. The emerging conclusion is that eradication is feasible in a relatively small number of cases (Panetta 2015; Scott et al. 2019; Williams et al. 2019; Hulme 2020), owing largely to attributes that distinguish weeds from other invasive organisms, such as the potential for development of persistent seed banks. Difficulties in achieving eradication are generally more pronounced for weeds of natural, as opposed to highly modified ecosystems (Panetta and Timmins 2004; Panetta 2009; Moore et al. 2011).

Given the constraints on the applicability of eradication as a weed invasion management goal, containment emerges as a logical management alternative should



**Figure 1.** Key species traits and other aspects that affect containment feasibility (modified from Grice et al. 2010). Traits with asterisks are employed in this piece to obtain a preliminary ranking of weeds for containment.

eradication not be feasible (Panetta 2009; Wilson et al. 2011; but see Fletcher et al. 2015). Containment can be either absolute (stopping spread) or relative (slowing spread), but the concept of absolute containment has extremely limited application (Panetta and Cacho 2012), often restricted to a scenario combining species that naturally spread slowly with the existence of strong barriers (Hulme 2006). Slowing spread can provide substantial benefits (Sharov and Liebhold 1998), including 'buying time' while more effective control methods, such as biological control, are developed. However, this strategy requires an indefinite commitment of funding and other resources. This is perhaps why there has been relatively little research on containment as a weed management goal (Grice 2009; Grice et al. 2010; Panetta 2012; Panetta and Cacho 2012; Fletcher and Westcott 2013; Fletcher et al. 2015). Multiple factors need to be considered in the estimation of containment feasibility (Fig. 1).

Following the recommendations made by Bartz and Kowarik (2019), herein we consider the context dependence of species impacts, as well as the prospects for successful management. We develop a simple method that combines weed risk with species traits that influence their capacity to spread, with the aim of producing a ranking of species that could serve as a key input for a more detailed evaluation of containment feasibility. For an example of the application of this method we focus on the weeds that threaten the plant communities found on Christmas Island, a small and isolated island

located in the Indian Ocean. These species are well established on the island, but many still have restricted distributions. Present resourcing constraints mean that eradication of even one of these species is not an option (A. Grigg, pers obs.), hence our focus on containment as a management goal.

# Methods

# The study area

Christmas Island lies in equatorial waters in the Indian Ocean, 370 km south of Java Indonesia and 1400 km northwest of Australia. The island is a submarine volcanic seamount that has been above the ocean surface for approximately 5 million years (Ali and Aitchison 2020). Total land area is 135 km<sup>2</sup>, most of which is greater than 150 m above sea level (ASL), forming a plateau; the highest point (Murray Hill) is 359 m ASL. The basalt core is mantled by a sequence of Eocene to Quaternary limestones that are often expressed as cliffs, scree slopes, outcrops and pinnacles (Barrie 1967; Grimes 2001). A few different soil types occur on the island, and they are predominantly derived from weathered substrate rocks (e.g. limestones or volcanics) or marine sediments accumulated during submergence phases. The depth of the soil profile is commonly less than 4 m but can range from zero (where limestone outcropping is present), to 80 m where fault or fissure infilling has occurred. Soils are often shallower on the geologically more recent lower terraces than on the central plateau.

The island has a tropical monsoonal climate, with most rainfall occurring between December and April. Long term average rainfall is 2200 mm pa, but this can be highly variable from year to year, ranging from 1067 mm (1987) to 5120 mm (2016) (Bureau of Meteorology 2020). Temperatures are very stable, with minimums of around 20 °C and maximums of around 29 °C. Relative humidity averages around 80%–85%. South-easterly trade winds dominate in the dry season (July to October), and north-westerly winds dominate in the summer wet season (Bureau of Meteorology 2020).

These conditions support a dense tropical rainforest across much of the island, with a canopy typically around 35 m and some emergent trees on the plateau approaching 50 m. The remote, isolated nature of this island has given rise to at least 253 unique species of fauna and flora, with endemic plants accounting for 17, possibly 18 species (Director of National Parks 2014; James et al. 2019). A total of 213 species of native plants has been reported (Claussen 2005). The Flora of Australia (1993) provides the most comprehensive description of vegetation types on the island, including seven old-growth/primary native vegetation types and two categories of secondary vegetation on historically cleared areas. Geoscience Australia (2014) developed a map showing vegetation and clearing patterns based on aerial images and LIDAR data (captured in 2011). A simplified form showing all five primary native vegetation types and historically cleared areas is shown in Fig. 2.



**Figure 2.** Main vegetation types and historical clearing boundary on Christmas Island (data courtesy of Geoscience Australia 2014).

Rainforest on Christmas Island is tallest over areas with deep soil (Flora of Australia 1993). In areas where limestone is nearer the surface (on the terraces), tree heights are reduced, the canopy is more open and diversity is often higher (Flora of Australia 1993). Forest on the plateau is evergreen with a closed canopy (>90% foliar cover) throughout the year. Vegetation on the terraced margins of the island is semi-deciduous (including both semi-deciduous forest and semi-deciduous scrub forest) and during long dry periods can become quite open (<50% foliar cover). This increases the invasibility of these forest types (Swarbrick and Hart 2000; Fine 2002; Green et al. 2004). Tree falls are common in all forest types, especially during wet season storms, and can provide opportunities for weed invasion.

The greatest disturbance factor, however, has been mechanised clearing for phosphate mining. Approximately 25% of the island's rainforest has been cleared for mining and associated developments since the island was settled in the 1880s (Fig. 2). As a quarry-type operation, large areas are cleared to remove the soil profile (~4 m), often leaving behind little or no soil on the pit floor. The post mining landscape is typically characterised by limestone pinnacles that can be deeply incised, sometimes standing over 5 m tall. Once equipment has dug out such areas, it can be impossible or too costly to access them again, meaning they can harbour weeds and be beyond control efforts. Only a small fraction of the total mined area can be rehabilitated sufficiently to restore even basic ecological functionality. Remediation works involve the use of heavy machinery, such as dozers, excavators, loaders and haul trucks, to rip compacted pit floors, break down limestone pinnacles and bring in what little soil is available from backfill stockpiles, generally at great expense. Early attempts at rehabilitation by mining companies in the 1970s involved planting a range of non-native tree species such as *Aleurites moluccana*, *Melia azedarach*, *Sesbania grandiflora*, *Syzygium jambos* and *Spathodea campanulata*, many of which have persisted to become weeds (Carew-Reid 1987). Since 1992, rehabilitation efforts have been carried out by the Australian Government's Christmas Island Minesite to Forest Rehabilitation program, using funds paid by the current mining company as a condition of its lease. New and old areas are being rehabilitated, with around 20,000 native trees planted each year. Weed control is a major component of the program.

Edges between rainforest and cleared areas, such as along roads, former drill lines for mineral exploration, railway lines and around mining pits, are extensive (Swarbrick and Hart 2000). Approximately 63% of the island is national park, 93% of which is old-growth primary forest (7851 ha) and the remainder is mostly rehabilitation fields. In addition, approximately 2337 ha of old-growth, primary forest is currently held on Unallocated Crown Land. Including pockets elsewhere, there is a collective total of ~10,215 ha of old-growth primary vegetation (all naturally occurring vegetation types). These virgin forest areas host the island's endemic and formally listed threatened species, and therefore are the priority for managing impacts from invasive species and human disturbance.

### Species-intrinsic weed containment feasibility

Panetta (2015) proposed a method for categorising plant species in terms of the species-intrinsic feasibility of eradication, based upon three attributes: 1) the time to reproduction (i.e., duration of the juvenile period); 2) the level of seed persistence; and 3) the potential for long distance dispersal (LDD), which has a disproportionate effect on the rate of spread (Nathan 2006).

In the case of containment feasibility, seed persistence is considered to be of secondary importance because the primary management focus is on the spread of a plant from a site rather than its persistence there (Wilson et al. 2017). The ability to contain a weed will depend therefore upon the potential for controlling its reproductive output, as well as the subsequent dispersal of whatever propagules are produced despite management actions (Panetta and Cacho 2014). Thus, the key determinants of containment feasibility are time to reproduction and the potential for LDD. A proxy for the latter is whether the dispersal suite (sensu Panetta 2012) is dominated by natural dispersal vectors such as wind, water or wild animals, or by humans and their agents. For the Christmas Island weeds under consideration, wind and wild animals (birds and bats) are the most active contributors to LDD (Swarbrick and Hart 2000). Potential reproductive output is another trait that can be considered in the determination of containment feasibility (Fig. 1). However, we do not address it here because a fundamental component of containment is the prevention of reproduction, even though some reproductive escape (Panetta 2007) may occur.

### Weed risk assessment

Consistent with the definition of risk assessment as threat (or hazard) × likelihood (Daehler and Virtue 2010), weed risk assessment involves an evaluation of species in terms of both their impact (threat) and their invasiveness (i.e., potential for spread, sensu Richardson et al. 2000). Various flora surveys over the past century, including several conducted during the last few years (by the Australian National Herbarium 2012 and 2019; Department of Agriculture, Water and Environment 2019; and Parks Australia), plus a review by Lohr et al. (2016), have identified more than 600 introduced species on Christmas Island. From this pool of introduced species, the whole-of-island weed risk was assessed for 130 species that had shown evidence of naturalisation. Personnel (n = 10) from Parks Australia, the National Herbarium, the Department of Agriculture, Water and Environment, and private consultants provided input that was compiled by Parks Australia to reach consensus. Many of the factors included in the Australian weed risk assessment model (Pheloung et al. 1999), such as potential adaptation to local conditions, weed history, plant type, undesirable traits, reproduction, dispersal mechanisms and persistence attributes were considered. More than 70 species were deemed problematic for secondary vegetation types, such as rehabilitation plots and other disturbed areas. Given that our focus here relates only to old-growth, primary vegetation types, species whose impact was restricted to secondary vegetation types were removed. This resulted in a pool of 31 priority species (Table 1) that had been assessed as 'high', 'very high' or 'extreme' risk, a ternary categorisation resembling that employed by Booy et al. (2017) to prioritise non-native organisms for eradication.

From these whole-of-island risk assessments, species were scored according to the risk posed to two major plant communities on the island: closed-canopy evergreen rainforest (on the deep soils of the island's central plateau) and semi-deciduous scrub forest (on the shallow soils of the coastal terraces). Among the natural, primary vegetation communities on the island, these two vegetation types represent the endpoints of a continuum of invasibility, with the former being less invasible than the latter and semi-deciduous forest representing a transition between the two (Swarbrick and Hart 2000). In general, pristine tropical rainforests have a low invasibility for most weeds, and only shade tolerant non-native species pose a threat (Fine 2002) – that is, until a disturbance (natural or anthropogenic) takes place and allows opportunities for invasion by a broader range of weed species. The exclusion of most weeds by tropical rainforests has been attributed to the fact that the great majority of species that are transported to such areas lack specific life history traits, most importantly shade tolerance, necessary for invasion in the absence of disturbance (Fine 2002).

Species designated 'extreme risk' were those considered to have the potential to transform (Richardson et al. 2000) at least one of the communities. These species were scored as '4'. Those posing a greater risk to one community than the other were designated as 'very high' risk (scored as '3') in the former and as 'high' risk (scored as '2') in the latter. In some cases, low recruitment, survival and growth had been observed in one of the communities (e.g., *Syzygium grande* and *Hevea brasiliensis* in semi-deciduous

**Table 1.** Weed risk assessment (WRA) and containment feasibility (CF) ratings for the 31 species considered to pose the greatest threat to two major plant communities on Christmas Island. WRA ratings are 4 (extreme); 3 (very high); 2 (high); and 1 (other). CF ratings are based on species attributes of juvenile period (scored as 1 for < 2 yr and 2 for > 2 yr) and potential for long distance dispersal (scored as 1 for species that are wind- or bird- and bat-dispersed and 2 for those whose dispersal occurs primarily through gravity or explosive dehiscence).

Growth	Species	Risk assessment rating		Containment feasibility rating			
form		Closed-canopy	Semi-deciduous	Juvenile	Dispersal	Total	
		evergreen rainforest	scrub forest	period			
Tree	Adenanthera pavonia	2	2	2	2	4	
Tree	Aleurites moluccana	2	2	2	2	4	
Tree	Castilla elastica	3	1	2	1	3	
Tree	Clausena excavata	4	3	2	1	3	
Tree	Delonix regia	2	2	2	2	4	
Tree	Ficus elastica	2	1	2	1	3	
Tree	Hevea brasiliensis	2	1	2	2	4	
Tree	Jatropha curcas	2	3	2	2	4	
Tree	Leucaena leucocephala	2	4	1	2	3	
Tree	Manihot glaziovii	2	2	2	2	4	
Tree	Melia azederach	3	3	2	1	3	
Tree	Piper aduncum	2	3	2	1	3	
Tree	Pithecellobium dulce	2	3	2	1	3	
Tree	Psidium cattleyanum	2	3	2	1	3	
Tree	Psidium guajava	2	3	2	1	3	
Tree	Spathodea campanulata	3	3	2	1	3	
Tree	Syzygium grande	3	1	2	1	3	
Shrub	Pluchea indica	2	3	1	1	2	
Shrub	Tecoma stans	1	3	1	1	2	
Vine	Antigonon leptopus	2	4	1	2	3	
Vine	Calopogonium mucunoides	2	3	1	2	3	
Vine	Centrosema pubescens	1	3	1	2	3	
Vine	Ipomoea cairica	1	3	1	2	3	
Vine	Ipomoea nil	1	3	1	2	3	
Vine	Macroptilium atropurpureum	1	3	1	2	3	
Vine	Mikania micrantha	3	4	1	1	2	
Vine	Mucuna albertisii	3	2	2	2	4	
Vine	Mucuna gigantea	3	2	2	2	4	
Vine	Mucuna pruriens	3	2	2	2	4	
Vine	Paederia foetida	2	3	1	1	2	
Vine	Tinospora crispa	2	3	1	2	3	

scrub forest and *Tecoma stans*, *Ipomoea* spp. and *Macroptilium atropurpureum* in the closed evergreen forest). These species were designated 'other' risk (scored as '1'), such that all lower categories of risk were combined.

*Chromolaena odorata* (Siam weed) is another species that could potentially transform semi-deciduous forest types. A small, but seed-producing population was discovered in 2010 (Dodd et al. 2012) and was immediately destroyed. Recruits, all of which were controlled, continued to emerge during wet seasons for seven years. Monitoring is continuing – extensive searches for this species by Parks Australia staff have been conducted at least twice per year, but it has not been detected in any other location. While exhaustion of the *C. odorata* seed bank at the known outbreak site has likely occurred, there remains a possibility that this weed remains undetected elsewhere, or could reinvade the island, most likely from the Cocos Keeling Islands (~970 km away) via sea or air transport. Should *C. odorata* be detected again on Christmas Island, it would be targeted for eradication so it is not considered further.

We reiterate that numerous species were excluded from the priority list because they are considered less of a threat to intact, virgin forest types, but following significant disturbance would possibly need to be brought back into consideration.

### Combining risk assessment with species-intrinsic containment feasibility

As in the system proposed by Panetta (2015), species were classified according to whether their juvenile periods were less than or equal to 2 years, or more than 2 years (scored as '1' and '2' respectively – the higher score aligned with greater containment feasibility). Species whose potential for LDD is high were scored as '1' and those for which LDD potential is relatively low were scored as '2', again with the higher score aligned with greater containment feasibility. Containment feasibility score totals therefore ranged between 2 and 4 (Table 1). Because our emphasis was on the characteristics of species that contribute to their spread potential, we did not include plant community invasibility in our estimates of weed containment feasibility.

Priorities for containment were obtained for individual communities by combining weed risk assessment ratings with the containment feasibility ratings for each species.

## Results

Four species (*Antigonon leptopus*, *Clausena excavata*, *Leucaena leucocephala* and *Mikania micrantha*) were categorised as posing extreme risk (risk assessment ratings of '4'), (Table 1). *M. micrantha* is relatively widely established across the island in cleared areas, forest rehabilitation fields, vehicle tracks and some walking tracks. It invades forest edges and gaps, posing a threat to all plant communities, and will likely invade future gaps since it is wind-dispersed and develops persistent seed banks (Brooks et al. 2008; Macanawai et al. 2018). *C. excavata* is a well-dispersed species that establishes in all forest types. It appears to be the most shade tolerant of the priority weeds identified here and can attain high densities within intact closed-canopy evergreen rainforest. What makes this species even more problematic is that a large proportion of its seedlings escapes the intense predation by land crabs, to which seedlings of most species, both native and nonnative, are prone (Green et al. 1999; Green et al. 2004). *A. leptopus* and *L. leucocephala* have major impacts in the semi-deciduous scrub forest – the first as a smothering vine limiting natural recruitment and the second by developing monocultures, in part due to its allelopathic leaf litter (Chou and Kuo 1986; Ahmed et al. 2008).

The distributions of weed assessment ratings for the two communities were different, as more species posed either 'extreme' risk or 'very high' risk to the semi-deciduous



Figure 3. Distribution of weed risk assessment ratings for 31 species posing the highest threat to major plant communities on Christmas Island. WRA ratings are 4 (extreme); 3 (very high); 2 (high); and 1 (other).

scrub forest than to the closed-canopy evergreen forest (Fig. 3). These differences reflect the fact that invasion is dependent upon disturbances that reduce canopy cover in the closed-canopy evergreen rainforest, whereas semi-deciduous scrub forest has a higher base level of invasibility because it is more open.

Priorities for containment are shown for potentially invasive species in the closed canopy evergreen rainforest (Fig. 4) and semi-deciduous scrub forest (Fig. 5). For both communities there is a substantial number of tied values in the listed priorities.

## Discussion

Virtue et al. (2001) designated the essential criteria for addressing the feasibility of controlling weeds as: 1) stage of invasion; 2) weed biology; 3) means of control; 4) cost of weed control; and 5) motivation of land managers. The management goal may be to eradicate a weed or to contain it spatially – sometimes collectively designated as 'co-ordinated control' (FAO 2011) – or it may be to maintain the abundance of a weed below an impact threshold (i.e., 'maintenance control'; Panetta and Gooden 2017). Different methods (e.g., Panetta and Timmins 2004; Booy et al. 2017) have been developed for evaluating eradication feasibility. These consider biological and ecological, socio-political, economic and operational factors specific to the context of an incursion.

The technical feasibility of co-ordinated control concerns the biological features and environmental context that, taken together, have a large effect on both the cost of control and the probability of management success (Cacho et al. 2006; Cacho and Pheloung 2007). The categorisation of species based upon the duration of the juvenile period, seed persistence, and dispersal characteristics (Panetta 2015) has provided a simple method for a first-pass determination of the species-intrinsic feasibility of eradication for a range of plant species (Panetta 2015). However, little guidance exists

Risk	Intrinsic containment feasibility	Species	Preliminary ranking	
	3	Clausena excavata	1	
4	2	Mikania micrantha	2	
		Mucuna albertisii	3	
	4	Mucuna gigantea	3	
		Mucuna pruriens	3	
3		Castilla elastica	4	
	2	Melia azederach	4	
	3	Spathodea campanulata	4	
		Syzygium grande	4	
		Adenanthera pavonia	5	
		Aleurites moluccana	5	
	4	Delonix regia	5	
		Hevea brasiliensis	5	
		Manihot glaziovii	5	
		Antigonon leptopus	6	
	3	Calopogonium mucunoides	6	
		Ficus elastica	6	
2		Jatropha curcas	6	
		Leucaena leucocephala	6	
		Piper aduncum	6	
		Pithecellobium dulce	6	
		Tinospora crispa	6	
	2	Paederia foetida	7	
		Pluchea indica	7	
		Psidium cattleyanum	7	
		Psidium guajava	7	
1	3	Centrosema pubescens	8	
		Ipomea cairica	8	
	5	Ipomea nil	8	
		Macroptilium atropurpureum	8	
	2	Tecoma stans	9	

Figure 4. Prioritisation for containment of weeds that are potentially invasive in the closed-canopy evergreen forest.

Risk	Intrinsic containment feasibility	Species	Preliminary ranking	
	2	Antigonon leptopus	1	
4	3	Leucaena leucocephala	1	
	2	Mikania micrantha	2	
		Centrosema pubescens	3	
		Clausena excavata	3	
		Ipomea cairica	3	
	4	Ipomea nil	3	
		Macroptilium atropurpureum	3	
		Piper aduncum	3	
		Calopogonium mucunoides	4	
		Jatropha curcas	4	
3		Melia azederach	4	
	3	Pithecellobium dulce	4	
		Spathodea campanulata	4	
		Tecoma stans	4	
		Tinospora crispa	4	
		Paederia foetida	5	
		Pluchea indica	5	
	2	Psidium cattleyanum	5	
		Psidium guajava	5	
		Adenanthera pavonia	6	
	4	Aleurites moluccana	6	
2		Delonix regia	6	
		Manihot glaziovii	6	
		Mucuna albertisii	6	
		Mucuna gigantea	6	
		Mucuna pruriens	6	
	4	Hevea brasiliensis	7	
1		Castilla elastica	8	
Т	3	Ficus elastica	8	
		Syzygium grande	8	

Figure 5. Prioritisation for containment of weeds that are potentially invasive in the semi-deciduous scrub forest.



**Figure 6.** Contribution of initial screening to weed risk analysis. Grey shaded elements are addressed in the present treatment. Note that some species may need to be eliminated from consideration owing to social considerations.

in relation to the estimation of containment feasibility (Grice 2009; Grice et al. 2010; Panetta and Cacho 2012; Wilson et al. 2017).

In the present exercise we have modified the eradication algorithm of Panetta (2015) to this end. The duration of the juvenile period determines the return time for control (Panetta 2007) and the dispersal mechanisms in play determine how readily new foci of infestation might originate from plants whose reproductive output is reduced but not entirely prevented, or plants that escape detection altogether (Panetta and Cacho 2012). The inclusion of actual juvenile periods of weeds on Christmas Island would likely reduce the number of ties in the current species ranking.

### Place of preliminary ranking in the estimation of weed containment feasibility

Consideration of the spatial distribution of each species, landscape features, management aspects and social considerations will be required for the more thorough weed risk analytical assessment. Our weed risk analytical screen is a key input to the analytical process (Fig. 6) because it encapsulates the risk posed by each species, as well as its potential for spread. The latter is largely determined by how quickly a species produces propagules, in conjunction with propagule adaptations that increase the likelihood of long distance dispersal.

# Exploring the relationship between weed risk and management feasibility

In our view the relative importance of these components of weed risk analysis requires further investigation. In prioritising species for eradication Booy et al. (2017) assumed a symmetric relationship, i.e., a species that was evaluated as posing a high risk in conjunction with medium feasibility of management should receive the same priority as one that posed a medium risk and high management feasibility. Taken to the extreme, the acceptance of this relationship means that a low risk species with high management feasibility would be accorded the same priority as a species manifesting the opposite combination, but we question the rationale of allocating scarce resources to the management of any species considered to pose a low risk. We maintain, on the other hand, that risk should have a higher weighting - given resourcing constraints, only the species that pose the highest risk should be considered for coordinated control, with prioritisation within this group informed by management feasibility (as shown in Figs 4 and 5 for weed risk analytical screening). We consider this assertion to have general application, not being restricted to the system under examination. A consequence of lower technical feasibility is that more resources will be required to achieve a given management goal (Panetta 2015; Wilson et al. 2017). In the present context, extremerisk species may still warrant containment even if projected management costs are high relative to those associated with species posing lower risk. This is a prime example of the application of the precautionary principle in risk management, as advocated recently by Strubbe et al. (2019).

# Setting subgoals for containment - the risk of false precision

Two fundamental issues must be considered when attempting to set subgoals for a containment program. The first relates to the counterfactual scenario (i.e., the rate of spread that would occur in the absence of intervention) against which the degree of spread reduction could be assessed. The second relates to the value of reducing this baseline spread rate.

Various models have been used to estimate spread rates of different organisms, including non-native plants (Higgins and Richardson 1996; Parry et al. 2013). Each modelling approach has its strengths and weaknesses, but key concerns include scale and complexity, the choice of theoretical versus empirically based models and, particularly relevant to the spread of plants, the existence of multiple dispersal pathways. Estimations of model and parameter uncertainty will be important for the purposes of decision making (Parry et al. 2013). It is likely that any modelled baseline spread for an invasive plant would be accompanied by wide confidence intervals, especially where the potential for long distance dispersal is a critical factor.
As was stated at the outset, slowing spread can provide substantial benefits. Net benefits of containment, which incorporate the costs of containment actions, can be identified through bioeconomics, i.e., the optimal management of renewable biological resources. Bioeconomic models can be used when only rough estimates of benefits and costs are available (Cacho 2006). However, any form of benefit/cost analysis of invasive species management is subject to biases that may lead to underestimation of costs and overestimation of benefits (Dahlsten et al. 1989; Myers et al. 1998) and therefore can be associated with "extremely wide confidence intervals" (Simberloff 2003, p. 251). The net value of a containment program (benefits accrued minus program costs) can be expected to vary with the degree of reduction of spread. The theoretical form of this relationship is a matter for speculation, but it is possibly non-linear, with increasing net benefits per unit spread reduction as absolute containment is approached.

Since the reduction of spread and its value can be expected to have wide confidence limits, it is important to be aware of the risk of false precision (Roberts 2017). In the first instance, the articulation of precise management goals (such as "70% reduction of spread") should be avoided. In the second, the degree of confidence in a measure such as the unit risk reduction (in terms of damages averted per dollar invested) might not be high, however valid such quantification might be from a theoretical perspective. Rather, given an acceptable estimate of containment feasibility, we suggest the way forward may be to adopt containment as an 'aspirational' objective and use measurements of spread over time (Panetta 2012) as a basis for deciding whether to continue a containment program or switch to an alternative strategy, such as asset protection (Auld and Johnson 2014). This is analogous to the approach that is taken with weed eradication, which is also essentially an aspirational goal. If over time an eradication program fails to demonstrate progress, this management goal may be abandoned (Panetta 2015). The goal of absolute containment has a discrete set of potential outcomes (spread is either stopped or it is not). For relative containment, however, a multitude of outcomes would meet the management objective - strictly speaking, any non-zero degree of spread reduction less than absolute containment. The determination of what is an acceptable level of spread reduction is clearly beyond the scope of this paper. As per the argument presented above, the output of any such determination is likely to be a range of values, the breadth of which would be related to levels of uncertainty in the counterfactual scenarios of weed spread rate and impact in the absence of management intervention.

#### Reducing invasibility by managing disturbance

The closed-canopy evergreen rainforest is intrinsically less invasible in the absence of disturbance, but at the same time hosts most of the island's endemic and formally listed threatened plant species. *Clausena excavata* is a rare example of a weed that can establish under an intact canopy and should be prioritised for control, whether via a formal containment program or in terms of asset protection. It has proven to be an invasive non-native weed in tropical forests elsewhere around the world (Viera et al. 2010; Roseleine and Suzuki 2012; Biswas and Das 2014). Growing slowly under low light

conditions, the species probably depends upon gaps created by tree falls to become reproductive. Seven other species are considered to pose a very high, and 17 species a high risk to the rainforest (Fig. 4).

Tropical cyclones have been relatively uncommon for Christmas Island, with roughly only one significant system impacting the island every decade or so. However, if cyclones become more intense and/or more frequent because of climate change, this could have major implications for the invasibility of the island's primary vegetation types, including closed-canopy evergreen forest. A cyclone in 2014 (TC Gillian) caused significant damage across the island, stripped much of the forest canopy and gave *M. micrantha* the opportunity to greatly expand its range into intact forest areas where it had never previously been able to establish. Given that this species has seed that can persist in the seedbank for up to seven years (Brooks et al. 2008; Macanawai et al. 2018), it will likely become more problematic if cyclones occur more frequently. That said, an effective biological control agent, the rust fungus Puccinia spegazzinii (Day and Riding 2019) has now been approved for release on the Australian mainland and may soon be available for use on Christmas Island, pending the outcome of a separate import approval process. When widely established, the rust should reduce the effort required to control *M. micrantha* by other means. This illustrates the containment benefit of 'buying time' until a more sustainable control measure becomes available.

The native forests of Christmas Island have been substantially cleared and fragmented by mining for phosphate minerals. Island ecosystems and island endemic species are notoriously vulnerable to impacts, declines and extinctions because of habitat disturbance and the associated compounding threats of invasive flora and fauna (D'Antonio and Dudley 1994; Lonsdale 1999; Denslow 2003; Gimeno et al. 2006; Vilà et al. 2006; Reaser et al. 2007; Baider and Florens 2011; Andrew et al. 2018; Heger and Andel 2019; Brock and Daehler 2020). In addition to more than 3,000 hectares of broad-scale clearing, approximately 500 km of mineral exploration drill lines have also been made in a systematic grid pattern across much of the island, predominantly through the closed-canopy evergreen forest on the plateau (Parks Australia 2009). Most of these drill lines were pushed through in the 1950s, 60s and 70s, before many of the weed species of current concern were widely distributed on the island. In the relative absence of problem weeds, drill lines once naturally regenerated by successional processes involving native species. The vegetation on old drill lines is often almost indistinguishable from primary forest. From an ecological perspective it has regained full ecosystem functionality, with mature trees offering bird nesting opportunities and an abundance of red crab burrows. The expansion of numerous weed species across the island in recent decades has significantly altered the ecological dynamics of colonisation and succession. Aggressive invaders such as C. excavata, M. micrantha, L. leucocephala and others now overgrow and out-compete native recruits and have been observed colonising recent drill lines and cleared areas. There are now many weeds that will colonise and dominate larger disturbed areas (Fig. 4). Furthermore, newly formed edges to intact vegetation enable weeds – especially the more shade tolerant species such as C. excavata - to encroach into primary vegetation. Left unchecked, there is little doubt

that weeds will dominate post clearing recruitment, cause major edge effects and progressively invade forest types on the island. This highlights the critical importance of minimising further forest fragmentation via all forms of anthropogenic disturbance as part of a holistic approach to weed risk management.

# Conclusion

Decisions concerning which weeds of natural ecosystems are targeted for control, as well as the type of control that is to be undertaken, need to be based on the degree of risk posed and the degree of difficulty that can be anticipated in attempting to manage it. Prioritisations of species for control need to be framed with reference to individual plant communities, since weed risk can be expected to vary according to the environmental context. Stringent limitations in the availability of resources for management generally imply that only the species that pose the highest levels of risk should be considered for co-ordinated control. We have shown that traits that contribute to the species-intrinsic feasibility of eradication may also be useful in assessing the feasibility of containment for species with restricted distributions, thereby assisting in the prioritisation of weeds to this management end. The development of a method for considering the spatial distribution of each species, landscape features, management aspects and social considerations is underway.

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REVIEW ARTICLE



# Advanced molecular-based surveillance of quagga and zebra mussels: A review of environmental DNA/RNA (eDNA/eRNA) studies and considerations for future directions

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#### Abstract

Sensitive methods, capable of rapidly and accurately detecting aquatic invasive species, are in demand. Molecular-based approaches, such as environmental DNA (eDNA) surveys, satisfy these requirements and have grown in popularity. As such, eDNA surveys could aid the effort to combat the colonisation and spread of two notoriously invasive freshwater mussel species, the quagga mussel (*Dreissena rostriformis bugensis*) and zebra mussel (*D. polymorpha*), through improved surveillance ability. Here, we provide a review of dreissenid eDNA literature (both grey and published), summarising efforts involved in the development of various assays for use in multiple different technologies (e.g. quantitative PCR, high-throughput sequencing and loop-mediated isothermal amplification) and sampling scenarios. We discuss important discoveries made along the way, including novel revelations involving environmental RNA (eRNA), as well as the advantages and limitations of available methods and instrumentation. In closing, we highlight critical remaining gaps, where further investigation could lead to advancements in dreissenid monitoring capacity.

#### Keywords

Assay, ddPCR, HTS, LAMP, metabarcoding, nuclear DNA, qPCR, veligers

## Introduction

Quagga (Dreissena rostriformis bugensis) and zebra (D. polymorpha) mussels are aquatic invasive species (AIS), known for imposing costly economic and ecological damage (Higgins and Zanden 2010; Nalepa and Schloesser 2013). These mussels belong to a phylogenetically complex genus composed of at least six species (Rosenberg and Ludyanskiy 1994; Gelembiuk et al. 2006; Son 2007; Graf and Cummings 2019). Although native to the Ponto-Caspian, quagga and zebra mussels (referred to herein as QM-ZM) have become problematic invaders in North America and Europe (Karatayev et al. 2007, 2015; Ram and Palazzolo 2008; Nalepa and Schloesser 2013; Matthews et al. 2014). They possess several attributes that have contributed to their invasion success. Attributes include prolific reproduction in which microscopic floating larvae (veligers) are released into the water column and easily bypass visual detection (Johnson and Padilla 1996; Stoeckel et al. 1997), secretion of byssal threads that allow firm attachment to numerous types of substrates and infrastructures (Berkman et al. 2000; Peyer et al. 2009), broad thermal tolerance (Locklin et al. 2020) and the ability to withstand transport, drying, low oxygen levels and minimal food conditions (Kinzelbach 1992; Ricciardi et al. 1995; Baines et al. 2007; Snider et al. 2014; Doll 2018). Their accidental introduction into the Great Lakes of North America most likely occurred via transoceanic ships (specifically, ballast water discharge), with ZM first observed in 1988 (Lake St. Clair; Hebert et al. 1989) and QM in 1992 (Lake Ontario; May and Marsden 1992). Since then, populations of QM-ZM have spread throughout much of the United States (US) and south-central to south-eastern Canada through contiguous waterways, though overland human transport has also contributed to introductions in disjunct locations (Johnson and Carlton 1996; Johnson et al. 2001, 2006).

In 2007, QM-ZM were detected for the first time in the western US within three lakes of the Colorado River Basin. This discovery - and others like it (e.g. QM-ZM detections near the headwaters of the Columbia River Basin in 2016) - indicated a westward extension of the North American invasion front and led to the development of several initiatives aimed at preventing, containing and controlling the continued spread of QM-ZM. Initiatives included the Quagga-Zebra Mussel Action Plan for Western Waters of 2010 (QZAP 2010), the 100th Meridian Initiative of 2011 (United States Fish and Wildlife Service 2011) and the Safeguarding the West Initiative of 2017 (United States Department of Interior 2017). These initiatives increased coordination and standardisation of preventative measures, actions, protocols and policies across different jurisdictions and agencies (including at the National, Federal, State and Tribal levels), with input from multiple stakeholders. All initiatives called for an increase in strategic surveillance, including proactive monitoring of high-risk water bodies. In the most recent initiative, environmental DNA (eDNA) surveys were listed as priority QM-ZM monitoring actions, hypothesised to improve surveillance through increased probability of early detection (United States Department of Interior 2017).

Environmental DNA is a term commonly used to describe genetic material deposited or shed into the environment by living organisms and can include both extracellular and intracellular DNA (Ficetola 2008; Jerde et al. 2011; Thomsen and Willerslev 2015). In its broadest definition, eDNA can also encompass DNA in the form of whole microscopic organisms (e.g. bacteria, viruses, phytoplankton, QM-ZM veligers) captured during environmental sampling (Pawlowski et al. 2020). The steps involved in an eDNA survey typically include: 1) sampling a habitat of interest (commonly, freshwaters or marine waters) where a target species is suspected to be present or has the potential to be present and then 2) subjecting the collected sample to sensitive molecular assays, specifically designed to detect DNA from the target species, if present. These assays are typically based on polymerase chain reaction (PCR), with accuracy of detection often subsequently confirmed via standard Sanger DNA sequencing of all, or a subset of, resultant PCR products. With eDNA, the accurate and reliable detection of a specific organism (i.e. detection confidence) requires adherence to quality assurance-quality control (QA-QC) measures, as well as the use of rigorously vetted, high-quality assays. Detailed discussions outlining necessary QA-QC for eDNA-based surveillance efforts, as well as guidelines for developing and validating eDNA assays, are available in Goldberg et al. (2016) and Klymus et al. (2020a).

The ability to detect and/or identify organisms within an environmental sample, as based solely on the DNA within that sample, is not new. In fact, eDNA techniques have been used in microbial and ancient DNA studies for more than two decades (for reviews, see Pawlowski et al. 2020 and Pedersen et al. 2015, respectively). The application of eDNA sampling for AIS surveillance purposes is, however, comparatively younger. It made its debut as a novel technique in 2008, when pond water eDNA samples proved useful for detecting invasive American bull frogs (*Lithobates catesbeianus*; Ficetola et al. 2008). Since then, eDNA sampling has been widely adopted as an AIS monitoring tool, often outperforming traditional survey methods for hard-to-detect aquatic species, including QM-ZM (e.g. De Ventura et al. 2017; Gingera et al. 2017; Sepulveda et al. 2019; Blackman et al. 2020a).

The genetic material of interest in most molecular-based surveys is DNA. Similar methods targeting RNA (eRNA) are emerging, however, with particular emphasis in ballast/bilge water AIS surveillance (e.g. Pochon et al. 2017). Recent QM-ZM evidence (Marshall et al. 2021) notably supports hypotheses (Barnes and Turner 2016; Cristescu 2019) that at least certain types of RNA degrade faster than DNA in environmental matrices. As such, eRNA may be an appealing complement to eDNA in that it potentially offers enhanced discrimination between AIS detections originating from contemporary sources (i.e. live/very recently alive, locally-present organisms) and AIS detections resulting from relictual, non-local or transient sources. Regardless of target (DNA vs. RNA), molecular-based surveys are extremely sensitive (e.g. lower limits of qPCR detection for QM-ZM can be as low as three gene copies per µl; Sepulveda et al. 2020a) and capable of detecting even minute amounts of target DNA/RNA. While this makes them susceptible to potential contamination (i.e. false positive detections), we reiterate that high-quality assays and careful adherence to QA-QC measures (in the field and in the lab) ensures detection confidence. Resultant robust specificity and sensitivity make molecular-based surveys particularly useful along invasion fronts

where QM-ZM abundance may be low. Early detections play a critical role in QM-ZM management, increasing the probability of eradication and, thereby, helping to prevent spread (Wimbush et al. 2009; Counihan and Bollens 2017).

Various molecular-based technologies and protocols have been employed in QM-ZM eDNA surveys and numerous publications exist detailing those efforts. Improvements in eDNA methods have been made along the way to overcome the challenges presented by complex and impure environmental samples. Methodological improvements include refined protocols for isolation and extraction of eDNA, enhanced reagents to combat PCR inhibition and more stringent primer design requirements (Wilcox et al. 2013; Hinlo et al. 2017; Lance and Guan 2020). Additionally, improved methods using highly sensitive platforms like quantitative PCR (qPCR), droplet digital PCR (ddPCR), high-throughput sequencing (HTS) and field-portable machines have been developed, allowing for the quantification, massive parallel sequencing and rapid-onsite surveillance of eDNA, respectively. While each approach provides some level of detection precision for QM-ZM eDNA (i.e. taking into consideration rates of imperfect, or false positive/false negative, detections) and, thus, some level of reliable inference as to the likely presence of QM-ZM, there are advantages/disadvantages that make each approach more suitable for different applications, questions and/or sampling schemes. As such, a review of the current knowledge and a synthesis of information regarding these various methods is needed.

Here, we provide a review of QM-ZM eDNA literature, discussing how knowledge (Table 1) and methodology has evolved over time (Fig. 1). We close by discussing critical scientific and applied gaps, which require additional attention or investigation to advance molecular-based QM-ZM surveillance and inferences therein. The review is intended for eDNA practitioners of all levels. It is consequently written to be understood by large audiences, including non-molecular experts (e.g. AIS managers) interested in implementing eDNA surveys. To aid readership understanding, we have provided a glossary of terms and common eDNA approaches (Table 2).

### Studies to date

In this section, we cover the history of the development and use of molecular-based methods for detecting the likely presence of QM-ZM in a sampled water body. The section is largely organised by technology type, with one sub-section dedicated to types of molecular targets (including eDNA vs. eRNA). The order follows the general progression in QM-ZM eDNA techniques, including associated advancements in eDNA knowledge and/or eDNA sampling methods.

Literature cited and reviewed was acquired in two ways. On 8 May 2020, we performed a Google Scholar search for relevant literature, using the following key words in combination with "quagga mussel", "zebra mussel" and/or "Dreissena": ddPCR, eDNA, environmental DNA, HTS, metabarcoding, NGS, PCR, qPCR, RNA. On 21 May 2020, we submitted a request for literature (to include unpublished documents and/or grey literature) from members of the Government eDNA Working Group **Table 1.** Summarised findings and important highlights from the reviewed quagga mussel and zebra mussel (QM-ZM) environmental DNA (eDNA) literature, demonstrating the evolution of eDNA methods and knowledge over time. We focus on insights gained via qPCR and HTS, as these two technologies have dominated QM-ZM eDNA endeavours and provided the vast amount of advancements.

Citation	Туре	Significant findings and other highlights			
Tucker (2014)	qPCR	Optimisation of extraction methods needed			
		Species-specific primers need developed			
Bollens et al.	qPCR	• qPCR multiplexing may negatively impact detection sensitivity, indicating importance of optimisation			
(2015)					
Peñarrubia et	qPCR	• Autumn sampling increases detection success, likely as a result of high veliger presence following spring-			
al. (2016)		summer reproductive season			
<u> </u>		Levels of infestation can be estimated using qPCR			
Amberg and	qPCR	• Designed 1 ZM-specific COI assay, where primers are QM-ZM generic, but probe is ZM-specific, with			
Merkes (2016);		specificity of assay tested against 27 non-target taxa			
(2019)		• Detection success increased when eDNA sampling occurred at greater depths and above soft substrates			
Gingera et al.	qPCR	• Designed 3 assays: 2 ZM-specific (CytB and COI), 1 QM-ZM generic (16S), with specificity of assays			
(2017)	-	tested against 10 non-target species			
		• qPCR multiplexing negatively impacts sensitivity			
		• Autumn sampling increases detection success perhaps due to spawning activity aftermath (veliger			
		presence)			
		• Spring sampling decreases detection success potentially due to winter QM-ZM die off and increased			
		dilution from snow-melt			
DeVentura et	cPCR,	• Similar performance of qPCR and conventional PCR (cPCR), but with cPCR potentially being less			
al. (2017)	qPCR	susceptible to false positives (due to low sensitivity)			
		<ul> <li>eDNA concentration in field samples correlate well with known mussel densities using qPCR</li> </ul>			
		• Recommended mesocosm experimentation to better understand how environmental variables and veliger			
<u> </u>	DOD	presence influence eDNA concentration estimations			
Sepulveda et al.	qPCR	• Multi-scale occupancy modelling indicated that a high probability of detection was possible with eDNA			
(2019)		surveys, regardless of season, when substantial and adequate sampling efforts were undertaken (14 to 54			
		• Summer compliant proved the meet officient and required the forwart replicates to achieve high probability.			
		• Summer sampling proved the most encient and required the rewest replicates to achieve high probability			
Shogren et al	aPCR	Fnyironmental variables as well as eDNA shed and decay rates complicate aPCR-based estimations of			
(2019)	qi ore	biomass/abundance			
Sepulveda et al.	aPCR	Round robin comparison of 5 OM-ZM-specific probe-based oPCR assays revealed high reproducibility			
(2020a)	1	and repeatability (i.e. reliability) in results across different eDNA labs, with the best performing assay			
		identified as DRE16S (QM-ZM specific, Gingera et al. 2017) and with DRE2 (ZM-specific, Amberg et			
		al. 2019) identified as potentially susceptible to false negatives			
		• Cautioned against estimating biomass, based on qPCR results; estimated DNA concentrations were			
		imprecise and inaccurate in spiked samples			
Marshall et al.	qPCR	Ratio of eDNA:eRNA useful for assessing time since deposition in controlled aquaria settings			
(2021)		• mRNA H2B represents a useful target for assessing recent (< 24 h) presence of live QM-ZM			
		<ul> <li>Multi-copy 16S and 18S rRNA represent useful targets for detecting low density QM-ZM</li> </ul>			
		Suggested observed patterns may be more complex in natural environments			
Blackman et al.	cPCR,	• Detection success was greatest with cPCR and qPCR, but with all DNA-based methods outperforming			
(2020a)	qPCR,	kick-net sampling (caveat: HTS utilised a universal metabarcoding primer not specific to QM-ZM)			
721 1	HIS	QM-ZM density and sampling distance impacts detection in eDNA surveillance efforts			
Klymus et al.	HIS	• Mollusc-specific 165 metabarcode designed			
(2017)		• H I S-based detection outperformed traditional surveys			
		• It is read counts correlated well with initial DIVA concentrations within mock community samples,			
Prie et al	HTS	Bivalve-specific 16S metabarcode designed			
(2020)	1115	· bivarie-specific 105 metabaleoue designed			
Marshall and	HTS	QM-ZM specific COI metabarcode designed			
Stepien (2019)		• Methods allowed for discrimination of QM-ZM, as well as assessments of relative abundance and genetic			
		diversity			
		• Aquaria trials indicated that biomass estimates were most accurate after QM-ZM had occupied tanks for			
		7–14 days			
		• QM-ZM biomass may be best estimated when eDNA samples are collected near the bottom of a			
		waterbody			



**Figure 1.** Evolution of quagga mussel and zebra mussel (QM-ZM) environmental DNA methods through time. Numerous technologies have been used to amplify and detect the DNA of QM-ZM contained within environmental samples. Technology types include nanoparticle-based methods (i.e. carbon nanotube or light transmission spectroscopy, CNT/LTS), conventional PCR (cPCR), droplet digital PCR (ddPCR), high-throughput sequencing (HTS), loop-mediated amplification (LAMP), quantitative PCR (qPCR) and comparative methods. Here, we can see that methods have evolved over time, with qPCR and HTS currently dominating the field and with ddPCR emerging.

(GEDWG). This North American-based working group is comprised of eDNA practitioners from federal, state, local and non-government institutions (e.g. universities), several of whom have conducted QM-ZM eDNA studies. In total, 23 documents were acquired from both avenues and included in this review.

# Early DNA studies using whole specimens

Molecular-based approaches have aided the effort to combat the colonisation and spread of QM-ZM by providing a mechanism for sensitive and reliable early detection. Initial endeavours began with a focus on the molecular identification of, and assessment of genetic diversity within, whole QM-ZM specimens collected from infested waters. Methods are reviewed in Marsden et al. (1996), but in short, these early studies used PCR-free, electrophoresis-based analyses (i.e. allozymes) to individually discriminate amongst morphologically similar (and sometimes unidentified) adult QM-ZM, most commonly collected via trawling. Soon after, conventional PCR-based

**Table 2.** Glossary of terms relevant to (and explained specifically for) environmental DNA (eDNA) applications. Terms are grouped according to different molecular targets, sources of DNA and technology types. Terms relevant to the validation of eDNA methods and common eDNA challenges are also provided.

Term	Definition			
Molecular targets				
eDNA	Environmental DNA. Genetic material found in an environmental sample (e.g. air, water, soil). Can include both extracellular DNA and intracellular DNA, DNA shed from dead or living organisms and sometimes DNA from whole microscopic greenism (e.g. muscel valiesr).			
PNA	Environmental RNA Similar to aDNA excent that RNA is the target molecule			
Sources of aDNA	Environmental KINA. Similar to eDINA, except that KINA is the target molecule.			
D 1: 1				
N 1 1	EDIVA from non-living sources, for example, from decaying carcasses or as trapped in sediments.			
INON-IOCAI	eDINA from another location deposited into the local environment by another source, such as a predator or via			
Transient	eDNA deposited by a target species no longer present in the system as with a migrating individual			
Extracellular	eDNA not encapsulated within a cell, sometimes also referred to as naked, membrane compromised or free-			
	floating DNA. Anticipated to degrade faster than intracellular eDNA.			
Intracellular	eDNA within a cell. Anticipated to degrade more slowly than extracellular DNA.			
mtDNA	Mitochondrial DNA. Circular DNA found within mitochondria. Common eDNA target, due to supposed high concentration and long persistence.			
nuDNA	Nuclear DNA. Linear DNA found within the nucleus of every cell. Less common eDNA target than mtDNA.			
	Abbreviations used elsewhere include nDNA, ncDNA.			
Technologies us	ed to amplify eDNA			
PCR	Polymerase Chain Reaction. Method used to amplify DNA in a cyclical pattern, typically involving three steps: denaturing (separates double-stranded DNA), annealing (PCR primers anchor to the target DNA region, if found within the sample) and elongation or extension ( <i>Taq</i> polymerase synthesises new DNA strands, complementary to the sequence downstream of annealed primers). Steps are achieved within a thermal cycler, using cyclical heating and cooling, where amplification is typically allowed to undergo 25 to 50 cycle iterations.			
cPCR	Conventional PCR. Conventional PCR is the oldest and simplest form of PCR. It provides end-point detection, where successful DNA amplification is observed (as bands in gel electrophoresis) upon completion of the reaction. For this reason, cPCR is often also referred to as end-point PCR. Amplified products often undergo Sanger sequencing to confirm the associated DNA sequence matches that of the intended target.			
qPCR	Quantitative PCR. PCR method that incorporates fluorescent chemistry to achieve real-time, quantitative detection of amplified DNA. Relative quantification is achieved via comparisons with standard curves.			
Sanger sequencing	Method used to read the nucleotide ("sequence") pattern within PCR amplicons (i.e. amplified PCR products). Often used to verify the identity of positive eDNA samples and to ensure amplified product represents the target organism.			
HTS	High-throughput sequencing. Also referred to as next generation sequencing (NGS). Method that allows for massive, parallel sequencing of numerous DNA fragments (i.e. PCR products). In eDNA applications, metabarcoding primers are often used to simultaneously generate amplicons for HTS.			
ddPCR	Droplet digital PCR. Advanced form of qPCR, in which absolute quantification is achieved by partitioning samples into individual droplets via water-oil emulsion technology.			
CNT/LTS	Carbon nanotube and light transmission spectroscopy. eDNA amplification and detection methods employing nanotube materials.			
LAMP	Loop-mediated isothermal amplification. A method in which DNA is amplified at a single temperature (as opposed to PCR, which requires cyclical changes in temperature). Requires a unique polymerase ( <i>Bst</i> , rather than <i>Taq</i> ) and the use of numerous species-specific primers (typically 6) to create the amplification loop.			
Oligonucleotide	Short, single strand of synthetic DNA/RNA. Commonly used in PCR.			
Primer	Oligonucleotide which complements and binds to target DNA/RNA in PCR, initiating amplification of a selected DNA/RNA fragment. Each PCR reaction requires at least two primers (or a set), typically referred to as the forward primer and the reverse primer.			
Probe	Fluorescently-labelled oligonucleotide used in qPCR to increase reaction specificity. Employed simultaneously with species-specific forward and reverse primers, targeting a third species-specific fragment within the intended amplicon. Creates the fluorescence in probe-based qPCR applications.			
Assay	In this publication, we use assay to refer to the primer and probe combination used in probe-based qPCR eDNA applications.			
Universal primers	Synonymous with barcoding primers. A primer set recognised for broad taxonomic coverage, capable of amplifying DNA from numerous different taxa. Frequently used for species identification purposes, but where DNA is often amplified from a single organism. Typically combined with Sanger sequencing.			

Term	Definition
Metabarcoding primers	Similar to universal (barcoding) primers, but specifically optimised for use in HTS amplicon sequencing ("metabarcoding"). Commonly used to amplify the DNA present in bulk and/or eDNA samples, resulting in many PCR amplicons representing numerous different taxa. Typically target shorter DNA fragments than universal (barcoding) primers.
Metabarcoding	An HTS application. The (simultaneous) sequencing of a PCR product containing a mix of amplified DNA fragments ("amplicons"), where the amplicons are generated using metabarcoding primers and represent the DNA of targeted organisms found within bulk and/or eDNA samples. Subsequent bioinformatic analyses are required to assess species composition.
Terms relevant t	o method validation
Mock community	An experimental sample in which the sample contains a mixture of target DNA templates at known concentrations and/or of a known composition. Sample is created to mimic the species composition present in environmental samples. Often used to evaluate the sensitivity and specificity of HTS metabarcoding primer pairs.
Spiked sample	An experimental sample in which target DNA (either tissue-derived or, more often, synthetic) is added at a known concentration. Spiked samples can be used at different stages of the eDNA workflow and are often employed to test the reliability of eDNA methods.
Quality Assurance- Quality Control (QA-QC)	A set of protocols, measures and guidelines to ensure quality eDNA results (including, reproducibility and repeatability). Please reference Goldberg et al. (2016) for a detailed list specific to eDNA surveys.
In silico	Method used to assess the specificity of eDNA primers and/or assays. Typically represents the first validation step, where primer/assay sequences for the target species are compared to sequences of non-target (and often related and/ or co-occurring) species using data available from DNA repositories (e.g. NCBI's Genbank).
In vitro	Method used to assess the specificity and sensitivity of eDNA primers and/or assays. Typically represents the second validation step, where PCR amplification is attempted for target and non-target species using primers/assays determined to be species-specific during in silico testing. DNA used in the PCR is often invasively collected (i.e. extracted from tissues).
In situ	Method used to assess the specificity and sensitivity of eDNA primers and/or assays. Typically represents the third (and final) validation step, where species-specific primers/assays passing in silico and in vitro testing are employed using eDNA samples collected from sites where the target species is known to occur and where the target species is known to be absent. Ensures that the assays work as intended, with positive detections in occupied sites and with no detections (i.e. false positives) in unoccupied sites. Success indicates that the primers/assays are ready for field application, where target species presence/absence is unknown.
Limits of detection	Abbreviated LOD. A measure of sensitivity. Required to reliably distinguish detections from non-detections in qPCR and ddPCR applications. LOD represents the lowest eDNA concentration at which 95% of technical replicates amplify (i.e. are detected), as based on a serial dilution of target DNA. False negative detections may occur at concentrations below the LOD. For relevant guidelines/discussions, see Bustin et al. (2009) and Klymus et al. (2020b).
Limits of quantification	Abbreviated LOQ. Determines precision of quantification (i.e. ability to quantify eDNA copy number). Lowest eDNA concentration at which samples can be reliably quantified using qPCR or ddPCR. Based on a serial dilution of target DNA, where the coefficient of variation is below 35%. Concentrations below the determined LOQ cannot be reliably quantified. For relevant guidelines/discussion, see Klymus et al. (2020b).
Challenges enco	untered
PCR inhibition	Reduction of DNA amplification efficiency during PCR due to presence of substances co-extracted from environmental samples (e.g. humic acids). PCR inhibition can contribute to imperfect detection and inaccurate quantification.
False negatives	Failure to detect eDNA of the target organism, even when the target organism is present in the sampled environment. Can be a result of, amongst other factors, eDNA methods exhibiting low sensitivity, inappropriately designed primers that fail to amplify DNA of target taxon, low tolerance to PCR inhibitors and/or poor sampling protocols (design, timing, replication).
False positives	Erroneous detection of the target organism when the target organism is absent from the sampled environment. Can be caused by amplification of non-target organisms (poor specificity of the assay) or by cross-contamination (poor QA-QC, lab and field protocols). For important nuances regarding the term "false positive", see Darling et al. (2021).
PCR primer/ amplification bias	Preferential amplification of DNA from more abundant species or of species whose DNA contains fewer mismatches to the primer sequence. Causes variation of amplification efficiency amongst taxa. PCR primer bias is especially problematic in HTS when using metabarcoding primers and leads to losses in detection sensitivity (i.e. false-negative results) for some species and/or the inability to quantitatively assess eDNA results.

Term	Definition		
Tag hopping	HTS sequencing issue in which sequence reads are mis-assigned to samples. In the HTS workflow, individual		
or swapping	samples (within a pooled sample) are identified by unique identifiers, called a tag or index, composed of short		
	nucleotide fragments which are appended to the ends of PCR products during library preparation; sometimes,		
	these unique identifiers get mismatched during preparation and/or during sequencing in a process called tag- or		
	index-hopping. As a result, sequence reads are matched to the wrong sample, confounding results and potentially		
	increasing the risk of false-positive detections. May be minimised by applying unique pairs of indexes ("dual		
	indexes"; one index for each end of template DNA) instead of only a single unique index for each sample.		
eDNA	eDNA is subject to biotic and abiotic factors which contribute to its degradation. Decay refers to the reduction		
decay	in detectable quantities of eDNA over time as a result of degradation. The rate of decay can impact eDNA survey		
	success and must be considered for interpretations beyond presence/absence.		

(hereafter, cPCR) techniques were used to discriminate juvenile specimens of both species which had been tentatively sorted, based on morphology (Claxton et al. 1997). Here, efforts relied on the commonly used mitochondrial DNA (mtDNA) barcode locus, *cytochrome c oxidase subunit I* (*COI*), using universal primers recognised for their broad taxonomic coverage (Folmer et al. 1994; Hebert et al. 2003). Subsequent work incorporated the design and testing of newly-developed, species-specific primer sets, unique to QM or ZM. These species-specific primers targeted a variety of QM-ZM genes (mitochondrial *COI* and 16S rRNA genes and a nuclear gene for 18S rRNA) and were used as diagnostic markers to discriminate amongst microscopic, larval veligers collected via plankton tows. The DNA analysed in all methods was acquired from whole QM-ZM veligers. Early methods required an initial step to pre-sort and individually isolate veligers found within the plankton tows (Claxton and Boulding 1998). Later methods skipped this step and utilised bulk, unsorted tow samples (Frischer et al. 2002; Ram et al. 2011).

These foundational studies provided the knowledge and methodology necessary for expedited, molecular-based dreissenid identification at all life stages, thereby circumventing the need for rare taxonomic expertise. Furthermore, PCR-based approaches were proving to be far more sensitive than more traditional techniques. For example, Frischer et al. (2002) developed a cPCR method (targeting a nuclear gene for 18S rRNA) that specifically identified and detected microscopic ZM veligers in bulk samples containing diverse and unsorted arrays of whole planktonic species. Not only was this cPCR-based method able to discriminate amongst numerous taxa (including other invasive bivalves, for example, QM and Asian freshwater clam, Corbicula fluminea), its detection ability was estimated to be "300 times more sensitive than cross-polarized light microscopy" (Frischer et al. 2002). Yet a decade later, Frischer et al. (2012) observed poor sensitivity using the same cPCR-based methods, where veligers often went undetected. Observations in Frischer et al. (2012) and Hosler et al. (2017) revealed that detection results, based on PCR, can be significantly impacted by expertise in molecular techniques, including familiarity with appropriate sample/DNA preservation methods. Nevertheless, these early findings, based on whole specimens, paved the way for more complex QM-ZM surveillance endeavours utilising eDNA sampling.

## Early eDNA-like studies

Moving beyond whole specimens and bulk samples, Carmon et al. (2014) demonstrated the successful detection of lab-manipulated, free-floating QM eDNA within water samples, culminating several years worth of research conducted by the same lab (e.g. see Keele et al. 2013 and protocols/references therein). Here, the authors used COI primers developed by Keele et al. (2013) and combined cPCR with first generation (i.e. Sanger) sequencing to confirm specificity of amplified PCR products. These early eDNA primers and others (e.g. Frischer et al. 2002), target relatively long DNA fragments (often >> 300 bp). Optimised guidance now suggests eDNA primers should target DNA fragments < 250 bp (e.g. Klymus et al. 2020a), as smaller fragments are more likely to be detected in highly degraded and/or eDNA samples (Thomsen and Willerslev 2015). Still, in this experimental study, eDNA-based detection was more sensitive than traditional detection methods (specifically, light microscopy), especially when veligers had undergone structural degradation (i.e. undergone a bead-beating process or exposure to an acidic solution) prior to eDNA sampling and PCR. Yet, the authors noted a high prevalence of false negatives in their experimental eDNA samples (Carmon et al. 2014), where mussels were known to be present, but their DNA not detected. These findings indicated that additional optimisations were needed for QM-ZM eDNA sampling, processing and analyses to achieve greater detection success.

# Conventional PCR

Moving out of the lab and into infested waters, Lance and Carr (2012) coupled cPCR (targeting 18S; Frischer et al. 2002) and confirmatory Sanger sequencing with a propidium monoazide (PMA) pre-treatment. Propidium monoazide is a photoreactive dye that binds to DNA and subsequently inhibits PCR, but is incapable of permeating cell membranes (Nocker et al. 2007; Bae and Wuertz 2009). Since PMA cannot infiltrate intact cells, it was predicted that PMA could assist in the targeted detection of DNA from whole veligers, as opposed to (extracellular, free-floating) eDNA shed from adult mussels. The results of this pilot study were positive in that capture and detection of ZM eDNA was successfully demonstrated using water samples collected from a known-infested, natural setting. However, PMA's ability to discriminate between whole veligers and extracellular eDNA was not confirmed (i.e. PMA results were inconsistent with expectations). The appeal of PMA has since waned. Evidence now suggests that eDNA shed from live organisms can contain a mixture of both membrane-bound DNA (i.e. intracellular) and extracellular DNA (Turner et al. 2014), making PMA treatment ineffectual for veliger-specific detection.

# Nanoparticle technologies: Carbon nanotube and light transmission spectroscopy

Most eDNA technologies require some form of PCR. This is because PCR is effective in amplifying minute amounts of DNA such that it can be readily detected in

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downstream analyses. However, some early eDNA studies aimed to eliminate reliance on PCR and thus improve rapid, on-site (in situ) QM-ZM surveillance in ballast and/ or harbour waters. These efforts focused on the application of novel DNA hybridisation methods and employed nanoparticle materials, using one of two relevant technologies, either microfluidic carbon nanotube chips (CNT, Mahon et al. 2011) or light transmission spectroscopy (LTS, Li et al. 2011; Egan et al. 2013, 2015; Mahon et al. 2013). Despite goals to be PCR-free, all publications incorporated cPCR as an initial step in QM-ZM eDNA detection, with most utilising universal invertebrate primers (Folmer et al. 1994) for amplification purposes. Detection of QM-ZM was met with mixed success. For example, in the most recent LTS publication, Egan et al. (2015) sampled waters known to be infested by both species, but could only detect QM. Follow-up publications, demonstrating improved detection and/or PCR-free CNT/ LTS advancements, have not emerged. A likely explanation is that costs associated with nanoparticle technologies are prohibitive to further development and widespread application. Or, perhaps PCR-free CNT/LTS endeavours failed to produce reliable QM-ZM detection results. Subsequently, CNT/LTS eDNA technologies have not been widely adopted. An alternative PCR-free strategy has more recently emerged for QM-ZM eDNA surveys and is described in a sub-section below, where loop-mediated isothermal amplification is detailed.

# Quantitative PCR

In contrast to the endpoint analyses of cPCR (Fig. 2), quantitative PCR (qPCR) employs fluorescent chemistry that produce DNA amplification curves which can be visualised or monitored throughout the reaction (Wittwer et al. 1997). With qPCR, there are two basic chemistry options: dye-based (e.g. SYBR Green) and probe-based (e.g. TaqMan; Heid et al. 1996). Fluorescence is achieved differently with each. Fig. 2 illustrates these basic differences, but for a more thorough review and relevant background information, see Arya et al. (2005). It is important to note that probe-based qPCR is often preferred for eDNA applications (Herder et al. 2014), as it achieves greater specificity through the use of a target-specific, fluorescently-labelled third oligonucleotide ("probe"), as opposed to dye-based qPCR which utilises a fluorescent chemistry that non-specifically binds to any double-stranded DNA present in the reaction, potentially producing false positive detections, if DNA from non-target organisms is amplified (Marmiroli and Maestri 2007).

In qPCR, fluorescence increases over the duration of the reaction and is reflective of the amount of DNA amplified at each cycle (Higuchi et al. 1992, 1993). Thus, qPCR amplification curves can be used to indirectly quantify the original amount (or starting concentration) of target-DNA present in an eDNA sample using comparisons with a standard curve (Takahara et al. 2012). These standard curves are generated from serially-diluted, known-concentration (and often synthetic; Conte et al. 2018) DNA templates (Fig. 2). One advantage of qPCR, then, compared to cPCR, is that it not only provides a mechanism for inferring the presence/absence of target taxa, it also provides the potential for estimating taxa abundance (i.e. relative density or biomass,



**Figure 2.** Detailed descriptions of common and emerging quagga mussel and zebra mussel (QM-ZM) environmental DNA (eDNA) amplification strategies. Quantitative PCR (qPCR) and high-throughput sequencing (HTS) represent the most commonly used technologies in quagga and zebra mussel eDNA studies. Droplet digital PCR (ddPCR), an advanced form of qPCR, is an emerging technique with popularity likely to increase due to its high tolerance of PCR inhibitors, improved quantification and observed sensitivity. Here, we detail the specifics of each technique, highlighting how detection and quantification occurs with each. Colours represent three hypothetical environmental DNA (eDNA) samples, at three technical (i.e. lab, amplification) replicates. Positive symbols represent eDNA detection. Negative symbols represent no eDNA detection. Conventional PCR (cPCR) is a foundational technology which gave rise to the other amplification strategies. It is no longer a common eDNA approach (due to low sensitivity), but we include it here for comparative purposes.

but not absolute numbers). Correlations between these metrics and estimated concentrations of eDNA have been found (e.g. Thomsen et al. 2012; Pilliod et al. 2013), even in QM-ZM (Peñarrubia et al. 2016; De Ventura et al. 2017; Marshall et al. 2021). Caution is advised, however, as eDNA concentration may not scale predictably with biomass (Mauvisseau et al. 2019; Shogren et al. 2019; Sepulveda et al. 2020a). For a review and meta-analysis of this issue, see Yates et al. (2019), where it is suggested that further refinement is needed for reliable eDNA-based estimations of abundance.

In an early attempt to develop qPCR markers for ZM, Tucker (2014) designed a probe-based *COI* assay and tested it for specificity against non-target QM and Asian clams (*Corbicula fluminea*, hereafter, *Corbicula*). In trials utilising cPCR, dye-based qPCR and probe-based qPCR, the author found the assay was not ZM-specific, but instead amplified both QM and ZM. Furthermore, detection success was noted to be inconsistent in lab-simulated, eDNA-like samples (reservoir water "seeded" with ZM veligers before DNA extraction). The author concluded that, in future studies, optimisation of eDNA extraction techniques was needed and emphasised the importance of developing species-specific primers to achieve QM-ZM management goals.

Moving into field-based qPCR detection, Bollens et al. (2015) designed two multiplexed, species-specific, probe-based qPCR assays for QM and ZM. A multiplexed reaction employs multiple assays in a single PCR and, with appropriate optimisation, enables the detection of either multiple species (by incorporating several species-specific assays) and/or multiple different loci targeting one species of interest (by incorporating several assays targeting different genes); the latter has been shown to increase eDNA detection success (e.g. Lance and Guan 2019). Both assays developed by Bollens et al. (2015) targeted the cytochrome B (CytB) locus of the mitogenome, but with one assay specific to ZM and the other specific to QM. These assays were intended for use in the Columbia River Basin (CRB), US, which has yet to be invaded by either Dreissena, but represents a highly-susceptible, regularly-monitored watershed. Interestingly, an additional eDNA assay for *Corbicula* (known to be established in the River Basin) was simultaneously developed and employed by the authors as a methodological positive control, thereby establishing the effective deployment of all phases of the eDNA survey. Experimental evaluation indicated that the Corbicula assay could not be run in multiplex with the QM-ZM assays, as this resulted in a loss of detection sensitivity for ZM. The Corbicula assay was, thereafter, run separately from the dreissenid assays. Asian clams were consistently detected across all sampled sites during the eDNA survey effort, but neither QM or ZM were detected.

To the best of our knowledge, the Bollens et al. (2015) *CytB* assays have yet to be tested in waters known to have QM or ZM infestations. The reason for this is unclear. Perhaps it is because details regarding assay specificity are missing, with no information provided as to how the assays were evaluated for amplification in potentially co-occurring, non-target species (e.g. in silico, in vitro). In contrast, greater emphasis was placed on QM-ZM specificity during the development of later-occurring probe-based QM-ZM qPCR assays (e.g. Amberg and Merkes 2016; Gingera et al. 2017; Sepulveda et al. 2019), garnering these more recent – and demonstrably more specific – assays greater popularity for deployment in recent surveys. The incorporation of probe-based qPCR chemistry and thorough testing for specificity are known to increase target-species detection precision, including reducing the risk of false positives and, thus, improve the reliability of eDNA results (e.g. Wilcox et al. 2013).

Meanwhile, some authors were employing dye-based qPCR methods. Peñarrubia et al. (2016) published a dye-based qPCR method targeting the nuclear, single-copy histone H2B gene in QM-ZM (Table 3). The method proved successful for indiscriminate detection of both mussels in Spanish lentic systems, with observed greater detection sensitivity than a simultaneously employed, traditional microscopy-based survey method. Furthermore, Peñarrubia et al. (2016) implemented a pre- and postspawning season sampling scheme which, when combined with the quantitative abilities afforded by qPCR, provided novel findings for the seasonal- and life-stage-specific dynamics of dreissenid eDNA. Specifically, results from their study indicated greater amounts of QM-ZM eDNA were present in the autumn season, which they attributed to increased veliger presence following successful summer spawning. The authors drew two important conclusions, both of which would influence the approaches and interpretations of later occurring surveys: 1) qPCR could be used to estimate levels of QM-ZM infestation via quantification of Dressenid eDNA and 2) QM-ZM eDNA sampling could be optimised by capitalising on spawning activity, where mass veliger concentrations provide ample sources of DNA.

Continuing with efforts to refine sampling protocols, Amberg and Merkes (2016) and Amberg et al. (2019) provided a comparison of multiple different strategies, including methods that employed sampling from different levels of the water column (surface, mid-water and near the bottom), sampling from waters overlaying either hard or soft substrates (where mussels may or may not settle, respectively) and sampling at two seasonal intervals. Samples were collected from across two different lakes, one where ZM was densely established and another where ZM was newly invaded. Relationships between eDNA and environmental covariates (i.e. depth and substrate) differed between the two lakes. Generally, though, findings indicated that the concentration of ZM eDNA increased with depth and decreased at suspected habitat (i.e. hard substrates). To explain this observation, the authors hypothesised that ZM eDNA drifts to and settles at deeper sections of the lake, where it is less susceptible to degradation and where softer sediments coincidentally exist. It should be noted that, while QM-ZM are filter feeders, their filtering activity does not appear to increase local eDNA degradation rates (Mächler et al. 2018).

The probe-based assay developed and used in Amberg and Merkes (2016; Amberg et al. 2019), was – according to the authors – the first of its kind to be validated for specificity to ZM. The assay, DRE2 (Table 3) combines a dreissenid-specific *COI* primer set with a ZM-specific probe. It has recognised utility throughout the Great Lakes Region, having been screened for specificity against 27 non-target fish and mussel species common to the area. It has been subsequently employed in a number of publications (e.g. Sepulveda et al. 2019, 2020a; Shogren et al. 2019), but with evidence to suggest it has relatively low sensitivity (potentially due to its low annealing temperature) and, thus, presents a risk for false negative results (Sepulveda et al. 2019, 2020a).

Three alternative – and high-performing (Sepulveda et al. 2020a) – probe-based assays developed by Gingera et al. (2017) have become some of the most prevalently used qPCR assays in North American QM-ZM eDNA surveillance efforts (e.g. Devlin

**Table 3.** Metabarcodes and assays proven effective for environmental DNA/RNA surveillance of quagga (*D. rostriformis bugensis*, QM) and zebra (*D. polymorpha*, ZM) mussels, narrowed to those employed and/ or developed in the last five years (since 2016).

Primer	Targets	Sequence (5' to 3')		
HTS metabarcodes	1			
(ordered by increas	ing specificity)			
Blackman et al. (2020a); Mychek-Londer et al. (2020) (originally developed by Geller et al. 2013; Leray et al. 2013)				
mICOIintF,	Metazoans	F: GGWACWGGWTGAACWGTWTAYCCYCC		
jgHCO2198	COI	R: TAIACYTCIGGRTGICCRAARAAYCA		
Ardura et al. (2017	) (originally developed by Geller et al. 20	)13)		
jgLCO1490,	Marine Invertebrates	F: TITCIACIAAYCAYAARGAYATTGG		
jgHCO2198	COI	R: TAIACYTCIGGRTGICCRAARAAYCA		
Brown et al. (2016)	) (originally developed by Zhan et al. 201	3)		
Uni18S	Crustaceans, Molluscs, Tunicates	F: AGGGCAAKYCTGGTGCCAGC		
	18S	R: GRCGGTATCTRATCGYCTT		
Klymus et al. (2017	7)			
MOL16S	Molluscs	F: RRWRGACRAGAAGACCCT		
	16S	R: ARTCCAACATCGAGGT		
Prié et al. (2020)				
Vene01	Bivalves	F: CSCTGTTATCCCYRCGGTA		
	16S	R: TTDTAAAAGCCGAGAAGACCC		
Marshall and Stepi	en (2019)			
COIA	QM-ZM	F: AGTGTTYTKATTCGTTTRGAGCTWAGKGC		
	COI	R: GAYAGGTARAACCCAAAAWCTWAC		
DYE-BASED qPCI	R primers			
Peñarrubia et al. (2	2016)			
H2B	QM-ZM	F: CGCGCGCTCCACTGACAAGA		
	H2B	R: CACCAGGCAGCAGGAGACGC		
De Ventura et al. (2	2017) (originally developed by Bronnenh	uber and Wilson 2013)		
DbuCOI3	QM	F: GGGGTTGAACATTATAYCCACCGTT		
	COI	R: AAACTGATGACACCCGGCACG		
DpoCOI3	ZM	F: GCTAAGGGCACCTGGAAGCGT		
	COI	R: CACCCCCGAATCCTCCTTCCCT		
Blackman et al. (20	020a) (originally developed by Blackman	et al. 2020b)		
DRB1	QM	F: GGAAACTGGTTGGTCCCGAT		
	COI	R: GGCCCTGAATGCCCCATAAT		
Marshall et al. (202	21)			
165	QM-ZM	F1: GTTAATAGCTGTGCTAAGGTAGC (long amplicon)		
	16S	F2: TGGGGCAGTAAGAAGAAAAAAAAAA* (short amplicon)		
	mtDNA, mt-rRNA	R: CATCGAGGTCGCAAACCG*		
		*Gingera et al. (2017)		
COI	QM-ZM	F: ATTTTATCTCTTCATATYGGGGGAGC		
	COI	R: CCAATAGAWGTRCARAACAAAGG		
	mtDNA, mt-mRNA			
18S	QM-ZM	F: AACYCGTGGTGACTCTGGAC*		
	18S	R: GTGTCTCATGCTCCCTCTCC*		
	nuDNA, nu-rRNA	*modified from Williams et al. (2017)		
H2B	QM-ZM	F1: CGCGCGCTCCACTGACAAGA* (long amplicon)		
	H2B	F2: TTGCCCACTACAACAAGCGA (short amplicon)		
	nuDNA, nu-mRNA	R: CACCAGGCAGCAGGAGACGC*		
		*Peñarrubia et al. (2016)		
PROBE-BASED al	PCR assays			
(where probes are l	abelled w a 5' fluorophore dye + 3' quend	cher)		

Primer	Targets	Sequence (5' to 3')		
Gingera et al. (2017)				
DRE16S	QM-ZM	F: TGGGGCAGTAAGAAGAAAAAAAAAA		
	16S	Probe: CCGTAGGGATAACAGC		
		Alt. Probe*: AAAGTTACCGTAGGGATAACAGCGTTATCG		
		R: CATCGAGGTCGCAAACCG		
		*developed by Devlin and Youngbull (2019)		
ZEBCOI	ZM	F: SCCTGCGATAGATTTTTTGATTTTA		
	COI	Probe: CGTGCTGGATGTCAT		
		R: GCAGAACAAAGGGACCCG		
ZEBCYT	ZM	F: CATTTTCTTATACCTTTTATTTATTAGTGCTTTT		
	CytB	Probe: TAGGTTTTCTTCATACTACTGGC		
		R: CGGGACAGTTTGAGTAGAAGTATCA		
Amberg et al. (2019)				
DRE2	ZM	F: TGGGCACGGGTTTTAGTGTT		
	COI	Probe: CGTCCTTGGTG		
		R: CAAGCCCATGAGTGGTGACA		
Sepulveda et al. (2019)				
DREQM	QM	F: CTCTTCATATCGGTGGAGCTTC		
	COI	Probe: CCCGGCACGTATATTTCCTCATGTT		
		R: CAAAGGCACCCGATAAAACTG		
LAMP primers				
Williams et al. (2017)				
QM-ZM	FIP: TGAAAGATACGTCGCCGGCGAACTCGTGGTGACTCTGGAC			
18S	BIP: TGCCTACC	CATGGTGATAACGGGTGTCTCATGCTCCCTCTCC		
	LF: GTGCGATCGGCACAAAGTT			
	LB: TAACGGGGAATCAGGGTTCG			
F3: GTTAGCCCAGACCAACGC		F3: GTTAGCCCAGACCAACGC		
		B3: CTTCCTTGGATGTGGTAGCC		
ZM	FIP: AGAGACAGGTAAAAACCCAAAAAACTAATTGATTGGTACCAATAATACTGAG			
COI	BIP: ATTTTGTTC	CAGCTTTTTAGGGAAGGAAAAATCTATCGCAGGGCC		
	LF: CGAGGGAAACCTATATCAGGAAGA			
	LB: GGATTCGGGGGTGGTTGAACC			
	F3: TAATGGGGGGATTCGGAA			
		B3: GCTCCCCCAATATGAAGAG		
QM	FIP: AAGAAGCT	CCACCGATATGAAGAGCCACCGTTATCCAGGATT		
COI	BIP: AGAACATGAGO	GAAATATACGTGCCCACCAATAGAAGTACAAAACAAAG		
		LF: ATGGCTGGCCCTGAATGCC		
	LE	B: GGGTGTCATCAGTTTTATCGGGT		
		F3: ATTTGGTGGGGGTTGAAC		
		B3: GGCTAAAACAGGTATTGCTAA		

and Youngbull 2019; Sepulveda et al. 2019, 2020a; Trebitz et al. 2019; Watts 2020; Marshall et al. 2021). These assays are commonly cited in literature as ZEBCOI, ZEB-CYT and DRE16S (Table 3), with ZEBCOI and ZEBCYT being specific to ZM and DRE16S generically targeting both QM and ZM. During development, all assays underwent thorough vetting for specificity to QM-ZM, with particular emphasis in the Great Lakes Region of North America, via trials with 10 native, non-target mussels. When originally deployed, Gingera et al. (2017) used these novel assays along an invasion front, where qPCR-based eDNA surveys were used for early detection purposes. Results provided positive eDNA detections (later confirmed via visual surveys) in high-

risk areas, where QM-ZM had either been previously eradicated and possibly recolonised or not yet documented. The authors employed a seasonal sampling scheme and, in agreement with Peñarrubia et al. (2016), also observed increased detection success during autumn months. A number of possible explanations were provided to account for lower detection success during the spring months, including increased dilution due to snow-melt and potential cold-season (winter) die-off. Support for the hypothesis that dilution plays a significant role in the success of QM-ZM eDNA surveys was later demonstrated by Trebitz et al. (2019). Akin to Peñarrubia et al. (2016), the increased detection success, observed by Gingera et al. (2017) in autumn, was attributed to QM-ZM life history, where whole-veliger presence, post-spring/summer spawning, likely contributes to ease of eDNA detection.

De Ventura et al. (2017) discussed how veliger presence in eDNA samples may impact the performance of different technologies and the ability to accurately quantify eDNA. The study compared the performance of cPCR and dye-based qPCR, employing two species-specific COI assays (DbuCOI3 and DpoCOI3; Table 3) developed by Bronnenhuber and Wilson (2013). Both eDNA methods appeared to outperform conventional survey techniques (i.e. kick-net sampling and scuba surveys), with similar levels of detection achieved in waters from previously invaded reaches and in waters at the edge of an invasion front. However, the authors concluded that cPCR was a more robust method and could potentially outperform qPCR by being less prone to false positives (due to cPCR possessing lower sensitivity than qPCR). Even so, the authors were able to demonstrate the utility and advantage of using qPCR. Here, De Ventura et al. (2017) found that eDNA concentrations (estimated via qPCR) were positively correlated with known mussel densities, indicating that QM-ZM eDNA concentrations can be linked to population densities via biomass (at least in some cases). The authors, however, recommended mesocosm experimentation to further investigate this relationship, stating that the concentration and quantification of eDNA may be influenced by several factors, including veliger presence, PCR inhibitors and environmental conditions.

As De Ventura et al. (2017) eluded, eDNA is subject to environmental factors that impact its transport, persistence and degradation (for reviews, please see Barnes and Turner 2016; Harrison et al. 2019). To better understand how these factors influence ZM eDNA surveys in lotic waters, as well as how they influence the interpretation of eDNA concentrations for biomass, Shogren et al. (2019) conducted an eDNA survey along a 7-km stretch of an infested river in Denmark during the non-reproductive season. Using DRE2 (Amberg and Merkes 2016; Table 3), the authors investigated the relationship between eDNA concentration and ZM density, while considering site characteristics (river physicochemical and hydrologic variables, including velocity, macrophyte cover, temperature, pH, substrate type, chlorophyll a and nutrients) and mussel eDNA shed and decay rates (Sansom and Sassoubre 2017). Results revealed complex relationships amongst variables, highlighting the difficulty in accurately estimating mussel biomass/abundance solely from eDNA quantitative

data. Specifically, Shogren et al. (2019) found a weak relationship between ZM density and eDNA concentration. A stronger relationship was observed between water velocity, nutrient concentration and the spatial distribution of ZM eDNA. The authors suggested these findings could be used to develop future sampling strategies, where the fate of eDNA may be best predicted using hydrological modelling (e.g. eDNA transportation models, such as in Carraro et al. 2018 and as more recently detailed in Carraro et al. 2020).

Using multi-scale occupancy modelling, Sepulveda et al. (2019) investigated how sampling strategies (specifically, intensity and timing) may impact eDNA detection success in North American QM-ZM surveys. Here, the authors used three assays: a newly-developed QM-specific CO*I* assay (designated DREQM and tested against 15 non-target taxa; Table 3), DRE16S (Gingera et al. 2017) and DRE2 (Amberg et al. 2019). Results indicated that, amongst filtered samples collected in June, July and October, the greatest sampling-to-detection efficiency was observed in July, when the reproductive season appeared to afford a higher probability of detection. Yet, the authors reiterated previous recommendations that sample replication plays a crucial role in the probability of detection and overall eDNA survey success (e.g. Ficetola et al. 2015; Furlan et al. 2016; Willoughby et al. 2016). With QM-ZM, a fairly large number of eDNA field samples were needed to achieve high levels of detection confidence, with  $\geq 27$  and 14 samples required, respectively, for June/October and July eDNA survey endeavours.

By 2020, it was clear that field-based methodological approaches (e.g. seasonal timing, replication etc.) impacted the outcomes of QM-ZM eDNA surveys. Yet, no study had compared the outcomes, based on assay choice. To remedy this issue, Sepulveda et al. (2020a) published a "double-blind, round-robin validation" for five of the most commonly used QM-ZM-specific, probe-based qPCR assays (DRE16S, ZEB-COI, ZEBCYT from Gingera et al. 2017; DRE2 from Amberg et al. 2019; DREQM from Sepulveda et al. 2019). In this study, filtered waters were collected from seven widely disjunct lotic and lentic locales in the US where QM-ZM infestations were either known or unknown. Samples were analysed across several labs and outcomes compared. Results were highly reproducible (i.e. consistent and, thus, reliable) across labs and largely across assays, with the following caveat: DRE16S outperformed all other assays, while DRE2 performed the least effectively (as previously mentioned, likely due to unusually low annealing temperatures). Although the authors acknowledged that using multiple assays could reduce the occurrence of false negative results (and, logically, improve overall survey power and accuracy), they cautioned against multiplexing the tested assays, citing findings from Gingera et al. (2017), which suggested that multiplexing decreased associated assay performance. Furthermore, Sepulveda et al. (2020a) noted imprecise and inaccurate eDNA quantification in spiked water samples (i.e. experimental samples containing known concentrations of target species synthetic DNA), suggesting additional caution is warranted when estimating biomass from water samples with low concentrations of eDNA. Such findings contribute to

the growing body of evidence that qPCR-based quantification may be less than precise for QM-ZM eDNA samples, which are consistent with findings across numerous taxa (Yates et al. 2019).

### Droplet Digital PCR

Droplet digital PCR (ddPCR; Hindson et al. 2011) is a technologically advanced form of qPCR, recognised for DNA quantification precision. With ddPCR, microfluidic circuits and oil-water interactions are employed to partition individual DNA molecules and qPCR reagents (e.g. polymerase, primers, hydrolysis probes, free nucleotides etc.) into individual oil droplets. During this process, tens of thousands of droplets are generated and each undergoes an individual PCR. The concentration of target DNA within a sample is calculated, based on the number of droplets that fluoresce at a set level (i.e. in which target DNA has undergone amplification) relative to droplets that do not fluoresce (and, hence, lacked target DNA). This approach is a direct and more accurate method for quantifying DNA than "analogue" qPCR (i.e. conducted on a standard qPCR instrument with DNA concentrations estimated using standard curves) and is less susceptible to inhibitor-induced false negatives when eDNA concentrations are very low (Doi et al. 2015), which is commonly the case. Recent grey literature details the novel application of ddPCR for QM-ZM eDNA surveillance.

In a pilot study, Watts (2020) used the QM-ZM specific assay DRE16S (Gingera et al. 2017) in conjunction with a modified *Corbicula* assay (Cowart et al. 2018) to survey for ZM and Asian clams. Filtered water samples were collected at docks and boat ramps at six lakes in the north-eastern US across the following ZM and *Corbicula* infestation gradient: absent (i.e. control site), recently eradicated, transient, non-viable, newly identified and known. For ZM, detection success varied amongst sampling sites and months; the greatest detection success was observed in lakes with larger populations and when sampling occurred during the month of May (i.e. spring). The latter finding contrasts with previous evidence which suggested mid-summer (July; Sepulveda et al. 2019) and/ or autumn sampling (Peñarrubia et al. 2016; Gingera et al. 2017) provided the best QM-ZM eDNA detection success. Observed discordance across studies may be due to differences in sampling effort, where replication has been shown to significantly influence the probability of QM-ZM eDNA detection across seasons (Sepulveda et al. 2019).

Devlin and Youngbull (2019), employing a newly-developed portable instrument, also reported on the use of ddPCR to detect QM-ZM eDNA. The authors used Gingera et al. (2017) DRE16S primers, but incorporated a novel probe (Table 3). Interestingly, during a survey for QM-ZM in Lake Mead (AZ and NV, US), the study discovered that QM-ZM eDNA could be detected in near real-time by directly assaying lake water (i.e. no filtration or centrifugation of water samples; no purification, isolation or concentration of eDNA). However, direct assay results may not be indicative of outcomes in other waters, as Lake Mead represents an extremely infested location where QM-ZM eDNA may be in atypically high concentrations (i.e. readily detectable without the need to concentrate).

### High-throughput sequencing

High-throughput sequencing (HTS) is a modern technology in which numerous targets (e.g. samples, genes, DNA fragments, species) can be simultaneously sequenced, generating greater amounts of DNA data in shorter time frames, all while reducing sequencing costs. In eDNA studies, metabarcoding approaches are often used alongside HTS (in a multi-step process) to rapidly and bioinformatically identify the DNA (i.e. species) present in an environmental sample (Fig. 2). During the first step, DNA is typically amplified using cPCR and taxonomically broad "universal" barcoding or metabarcoding primers (Hebert et al. 2003; Taberlet et al. 2012). The obtained amplicons are subsequently sequenced or "read" via HTS and the resulting sequence data are then cross-referenced against either an existing DNA database (i.e. NCBI's GenBank, Barcode of Life Database (BOLD)) or a custom made DNA database. Using these databases and complex bioinformatic analyses, sequences (i.e. amplified DNA) can then be identified to species or higher taxonomic levels depending on the quality and taxonomic comprehensiveness of the reference databases. Metabarcoding presents unique challenges to eDNA analysis and interpretation. It is subject to losses in detection sensitivity (e.g. PCR amplification bias), increased risks of false-positive results (e.g. via contamination induced by HTS tag-hopping) and requires robust experimental evaluation (Zinger et al. 2019).

Metabarcoding HTS methods have been successfully applied to QM-ZM eDNA surveillance efforts, where several surveillance objectives have been met using a variety of primers (Table 3). For example, COI metabarcoding primers, designed to generically target metazoans (Leray et al. 2013) and/or marine invertebrates (Geller et al. 2013), have been used to specifically detect ZM (Ardura et al. 2017) or QM eDNA (Blackman et al. 2020a) and to detect QM-ZM eDNA in community-wide surveys (Mychek-Londer et al. 2020). Metabarcoding primers targeting 18S and designed to detect crustaceans, molluscs and tunicates (Zhan et al. 2013), have also been used alongside HTS to detect AIS at freshwater ports, revealing the presence of QM (Brown et al. 2016). Blackman et al. (2020a) report two important HTS findings from knowninfested waters: 1) an increase in distance between sampling (i.e. the point of eDNA collection) and the source population negatively influenced QM eDNA concentrations and 2) HTS underperformed in comparison to simultaneously employed speciesspecific cPCR and qPCR (Table 3), where QM detection was 86% and 100% successful, respectively. The latter finding was especially true in low density populations. The observation that HTS was less sensitive than species-specific qPCR is consistent with other studies (Lacoursière-Roussel et al. 2016b; Harper et al. 2018; Bylemans et al. 2019). Nevertheless, the relatively low performance of HTS in Blackman et al. (2020a) may be due to the use of a universal primer that presumably targets most animal groups (metazoans), but was not specifically designed for molluscs and, as a result, may preferentially amplify DNA from other, more abundant species and/or other species whose DNA exhibits better matches to the primer sequence. Thus, in the following paragraphs, we detail the development and use of more-specific HTS metabarcodes

(e.g. mollusc-specific, bivalve-specific, QM-ZM-specific), which may provide better QM-ZM eDNA results.

Klymus et al. (2017) developed metabarcoding primers for specific use in AIS HTS efforts targeting molluscs (i.e. bivalves - or mussels and clams - and snails). To begin, the authors investigated the discriminatory power of three DNA regions (mitochondrial COI and 16S and nuclear 28S) to detect and discriminate 19 invasive/potentially-invasive snail and bivalve species of concern within the Great Lakes Region. The most suitable region was 16S. Of the two primer sets subsequently developed, MOL16S (Table 3) was intended for use in molluscs, including QM-ZM. Performance of MOL16S was first evaluated using an experimental "mock community" eDNA sample, in which a solution was created containing a mixture of targeted DNA templates at known concentrations. The authors assessed the interaction of PCR amplification bias and amplicon/sequence read abundance. Amplification bias is described as the tendency of a primer to preferentially amplify ("detect") the DNA of certain species over others. The bias, which can impact HTS results, is largely due to nucleotide mismatch between the DNA sequence of the primers and the complimentary DNA regions of different targeted species (Piñol et al. 2014). The authors found that the number of observed sequence reads for a species correlated well with initial DNA concentrations. Thus, in eDNA surveys, HTS data may be useful for semi-quantitative purposes, providing rough estimations of the relative abundance/biomass of a target species. When the authors later deployed their technique in Great Lakes waters, MOL16S proved to be less specific than anticipated and, additionally, amplified DNA from non-targeted groups including oligochaete worms, rotifers and bryozoans. However, because sequences from different species can be parsed within HTS, the presence of QM-ZM could still be discerned, with the HTS eDNA effort still outperforming visual surveys. Snyder et al. (2020) later used HTS and MOL16S to successfully monitor for QM-ZM in holding-tank waters in bait shops in the Great Lakes Region.

Prié et al. (2020) also developed bivalve-specific HTS metabaroding primers, targeting 16S in the orders Unionida (Unio01) and Venerida (Vene01). Primer Vene01 (Table 3) was designed such that members of the Dreissenidae family, including QM-ZM, would also be amplified and detected. Field samples from predominantly French lotic systems proved that Vene01 could successfully detect QM eDNA. The HTS primer provided evidence supporting a wider distribution and expanded invasion, for QM in that region.

Even greater metabarcoding specificity was achieved in Marshall and Stepien (2019). Here, the authors developed two HTS CO*I* primer sets (COIA and COIB), which were designed to detect QM-ZM, as well as four other *Dreissena* species. The primers were additionally useful in discriminating amongst haplotypes within those species. Experimental tests, based on mock community samples, showed that COIA (Table 3) outperformed COIB. Consequently, the authors solely present HTS results from aquaria trials and field sampling using COIA. Ultimately, the authors were able to successfully assess QM-ZM species composition, relative abundance and population genetic diversity using eDNA samples and their newly-developed HTS method. Not

only were HTS read counts for the two species well-correlated with known QM-ZM biomass, the approach also produced QM and ZM haplotype reads proportional to the haplotypic representation found in local populations of the two species. The aquaria trials produced two interesting results concerning HTS-based biomass estimations. First, HTS read abundance best matched known biomass after an acclimatisation period of 7–14 days. Second and similar to field-based findings in Amberg et al. (2019), aquaria trial evidence from this study further indicated that QM-ZM biomass may be best estimated using water samples collected near the bottom rather than at the surface.

#### Loop-mediated isothermal amplification

Technologies like cPCR, qPCR, ddPCR and HTS all achieve DNA amplification via thermal cycling and, thus, require instruments capable of rapid, cyclical heating and cooling. This is a significant limitation for in situ eDNA surveys, especially eDNA surveys in remote, inaccessible locations where it may be difficult to transport and power thermal-cycling equipment. A more field-friendly option - capable of providing point-of-collection results (Stedtfeld et al. 2012) and, thus, minimising delays in AIS surveillance (Merkes 2020) - may be found in loop-mediated isothermal amplification (LAMP; Notomi et al. 2000). Here, amplification occurs at a single temperature using a unique polymerase and three sets of specially designed primers, termed forward and backward inner primers (FIP and BIP), loop primers (LF and LB) and outer primers (F3 and B3). The unique polymerase, used in LAMP, is highly tolerant of amplification inhibitors (Koloren et al. 2011). This attribute makes LAMP appealing for eDNA samples, where inhibitors are especially challenging and can lead to amplification failure in PCR-based techniques. Yet, studies have found LAMP assays to be roughly 10× less sensitive than qPCR (Bühlmann et al. 2013; Waliullah et al. 2019) and may be unable to detect the very low concentrations of DNA typically observed in eDNA samples. Increased sample volume may provide a trade-off here. For example, with LAMP, potentially larger (and dirtier) volumes of water can be processed (i.e. filtered and extracted), without losing sensitivity due to inhibition. This could potentially allow for an increase in the capture and concentration of available eDNA and, thus, improve LAMP-based detection probability.

Williams et al. (2017) is the only publication to have successfully demonstrated, via lab and field trials, a QM-ZM eDNA LAMP capability. The authors developed three novel assays (Table 3) and investigated whether filtration and subsequent DNA extraction impacted the sensitivity of their LAMP-based approach. These are important investigations because LAMP is often used to directly amplify "crude" samples with minimal (if any) pre-processing (e.g. Stedtfeld et al. 2014; Maranhao et al. 2020). However and as mentioned above, eDNA samples typically undergo filtration (and subsequent DNA extraction) before amplification. These steps serve to concentrate target DNA, but coincidentally concentrate inhibitors as well. Performance was evaluated across sites with known and variable levels of QM-ZM infestation (i.e. high- vs. low-density populations) and where eDNA samples were collected across multiple seasons

(including spawning season). In high-density situations (i.e. in large populations or during the spawning season), the authors found that neither filtration nor extraction was required to successfully detect QM-ZM using LAMP. In fact, the authors demonstrated that direct and PCR-free amplification of QM-ZM eDNA could be achieved at the point of collection in less than 90 min using a handheld, battery-operated LAMP device (Gene-Z; Stedtfeld et al. 2012). However, the greatest detection sensitivity was achieved when eDNA samples underwent both filtration and DNA extraction before being amplified with LAMP. This was particularly true for low-density populations, where filtration likely helped to concentrate DNA. Inhibition did not appear to be problematic for LAMP-based QM-ZM detection in these situations. Despite these successes, the incorporation of filtration and DNA extraction steps may decrease the field-friendliness of LAMP by requiring transport of additional equipment and reagents.

## Target type

The vast majority of eDNA sampling endeavours, especially those involving QM-ZM, have relied on assays targeting short fragments of mtDNA (but see, Lance and Carr 2012; Peñarrubia et al. 2016; Williams et al. 2017). Mitochondrial DNA has dominated the field for two main reasons. One reason is that vast amounts of online sequence data exist for mtDNA. These readily accessible data make it easier to design effective eDNA assays that meet criteria for inclusivity (detect all genetic variants of target locus in species) and specificity (detect target taxa only). A second reason mtDNA is so popular in molecular-based surveys is that multiple lines of evidence have led to generalisations that multi-copy, membrane-bound mtDNA exists in the environment at higher concentrations and for longer periods than single-copy nuclear DNA (nuDNA) and/ or single-copy RNA (Thomsen and Willerslev 2015). Evidence also exists to suggest the same trends occur for short molecular fragments as compared to long molecular fragments (e.g. Jo et al. 2017). Ultimately this means that short mtDNA targets are appealing for use in eDNA endeavours because they are easier to detect. Yet, if lower concentration, longer fragments are subject to greater degradation and decay, perhaps these types of targets provide greater potential to selectively detect more contemporary signals, thus providing stronger indications that a living individual was recently present in the sampled system (Barnes and Turner 2016; Bista et al. 2017; Cristescu 2019). Growing evidence, however, reveals that, across these various types of molecular targets, patterns in deposition and degradation (which, in turn, influence abundance, persistence and detectability) are more complex than previous generalisations would suggest (e.g. Bylemans et al. 2018b; Harrison et al. 2019; Wood et al. 2020). Still, interest remains in how each of these unique molecular targets can be used, individually and in complement, to address various different surveillance objectives, including improved spatio-temporal inferences regarding distribution and time since deposition (hereafter, age). Very recent evidence provided by Marshall et al. (2021) suggests more accurate estimates of age can be achieved in molecular-based QM-ZM detection signals when surveys simultaneously employ both eDNA and eRNA.

In fact, Marshall et al. (2021) investigated a number of pertinent questions related to the use of various molecular targets in qPCR-based QM-ZM detection and revealed profound new insights. Here, experiments were conducted in which aquaria samples were analysed for a combination of six different QM-ZM molecular targets (Table 3). These targets represented both the nuclear and mitochondrial genomes, and allowed for comparisons of different fragment lengths (i.e. short vs. long), eDNA vs. eRNA and messenger RNA (mRNA) vs. ribosomal (rRNA). To assess patterns in abundance, degradation and detectability across time, aquaria sampling occurred at intervals covering 0 and 4-240 h after QM-ZM removal. All targets were analysed, separately, as eDNA and as eRNA. To obtain separate eRNA data, eRNA extraction methods were used, with an additional reverse transcription PCR step added to the typical eDNA workflow. Marshall et al. (2021) concluded that they were better able to estimate the age of environmental genetic material when they combined both eDNA and eRNA and investigated the ratio of degradation between the two. Other important observations include: 1) similar to eDNA, eRNA concentrations can be positively associated with QM-ZM abundance across nuclear and mitochondrial genomes, 2) decay constants were similar for short (75-169 bp) vs. long (251 and 341 bp) gene targets/ fragments, 3) multi-copy rRNA genes (mitochondrial 16S rRNA and nuclear 18S rRNA) may improve detection in low density situations due to observed higher concentrations (and longer persistence after QM-ZM removal) and 4) mitosis-associated H2B mRNA provides a useful eRNA target for assessing the recent presence (< 24 hrs) of live QM-ZM.

These QM-ZM specific experimental findings are in contrast to those of Wood et al. (2020). They conducted similar aquaria-based decay rate experiments in another AIS (a marine polychaete worm) and found (using ddPCR) that eRNA only remained detectable in aquaria samples within 14 h of target-organism removal, while eDNA persisted for much longer (up to 94 h after organism removal). Importantly, however, Wood et al. (2020) attributed these differences to initial eDNA/eRNA concentrations (i.e. shed rates), as opposed to any difference in decay rates, which were not found to be significantly different. Still, in both Marshall et al. (2021) and Wood et al. (2020), eRNA was found to persist at unexpectedly long intervals. For QM-ZM eRNA targets, the decay rates (presented as model-derived, log-linear per hour constants) ranged from -0.0561 to -0.0735 (± 0.0025), equating to eRNA half-lives between 8.84 to 13.54 h (Marshall et al. 2021). It is possible that the experimental aquaria lacked natural bacterial communities and perhaps this helps explain the unexpectedly long intervals of observed eRNA persistence. Bacterial communities are known to contribute significantly to eDNA degradation (Nielsen et al. 2007; Lance et al. 2017, Zulkefli et al. 2019; Saito and Doi 2020) likely via enzymatic and metabolic activity (Finkel and Kolter 2001; Vorkapic et al. 2016; Al-Wahaibi et al. 2019). Thus, observed results may not reflect the rate of decay in natural systems where degradation may be expedited and/or more severe and/or where environmental conditions may further influence the fate of different molecular targets (e.g. see Harrison et al. 2019). Differences aside, evidence from Wood et al. (2020) suggests that tank biofilms may act as accumulators

of "legacy" eDNA and eRNA (detectable up to 21 days post-organism-removal). This has important implications for QM-ZM monitoring efforts, especially those focused on assessing successful decontamination of ballast/bilge tanks. If legacy genetic material exists in biofilms of decontaminated tanks, this may lead to positive detections of QM-ZM across extended periods of time, even when QM-ZM are no longer present and/or viable.

# Summary, including paths forward and critical remaining gaps

A robust suite of sensitive molecular-based methods has been used to successfully monitor invasive QM-ZM in North American waters and elsewhere. As such, more than 20 QM-ZM eDNA reports (in both peer-reviewed and grey literature) were reviewed herein, spanning a decade's worth of research, development and implementation. Approaches for eDNA-based QM-ZM surveillance have evolved from simple cPCR to cutting edge ddPCR and HTS (Fig. 1). Although eDNA sampling has been the pioneering focus of most molecular-based QM-ZM monitoring endeavours, recent eRNA findings suggest that exciting new avenues are on the horizon, in which eDNA and eRNA can be used together to advance spatio-temporal inferences. Despite much progress (Table 1), a number of critical scientific and applied gaps require resolution. We close this review by discussing ways in which the QM-ZM field can move forward by utilising existing recommendations for optimised best practices, while also highlighting critical remaining gaps in need of attention.

# Assay/Metabarcode choice

Detection success and accuracy of results, can depend heavily on assay and/or HTS primer choice (e.g. Wilcox et al. 2013; Elbrecht et al. 2019). Although a wide variety of assays and primers are available for use in molecular-based QM-ZM survey efforts (Table 3), variable levels of validation and efficiency have been reported for each. A consensus to utilise only the best (i.e. most specific and sensitive) of these assays would benefit QM-ZM management and surveillance programmes by making results not only more reliable, but also more directly comparable. Findings from Sepulveda et al. (2020a) can be used as a guide in this respect, assisting future surveyors in the right direction for probe-based qPCR assay selection. Assay selection could be further assisted by the evaluation criteria recently outlined in Thalinger et al. (2020). Here, assays designed by Gingera et al. (2017), De Ventura et al. (2017) and Williams et al. (2017) have already been objectively identified as reliable. According to the supplementary data in Thalinger et al. (2020), the assays reached levels 3 and 4 out of a 5-level rating scale, in which minimum criteria covering 14 basic lab and field validation steps had been "essentially" or "substantially" demonstrated. This rating ultimately means that these assays are ready for field application, but are not fully operational and, thus, may limit the interpretations of non-detection results (e.g. at level 3, it is impossible to tell

if negatives are false-negatives) and/or may require additional steps to validate positive detections (e.g. sequencing amplicons from positive samples).

Based on Sepulveda et al. (2020a) and Thalinger et al. (2020), we recommend the use of DRE16S (Gingera et al. 2017; Table 3) as a first choice for qPCR-based QM-ZM eDNA surveillance. We note, however, that the use of multiple assays targeting different gene regions improves detection success and accuracy (Lance and Guan 2019; Sepulveda et al. 2020a, b). Surveyors employ additional (non-16S) assays from the list of reliable choices in Sepulveda et al. (2020a) and Thalinger et al. (2020).

To our knowledge, similar evaluation criteria do not exist for HTS metabarcodes (but see, for example, methods used in Bylemans et al. 2018a and Elbrecht et al. 2019). Comparative evaluations of available QM-ZM-relevant metabarcoding primers are still needed. As such, round-robin tests akin to those in Sepulveda et al. (2020a) should be used to identify the most reliable and effective metabarcoding primers for HTS-based QM-ZM eDNA endeavours.

#### Sampling effort

As with all AIS survey methods, molecular-based surveys are susceptible to imperfect detection. Field and lab replicates are known to improve eDNA detection probabilities (Ficetola et al. 2015; Furlan et al. 2016; Willoughby et al. 2016). Sepulveda et al. (2019) found this to be true in QM-ZM, where occupancy modelling indicated that substantial field sampling is required to obtain high probabilities of detection, but with variable effort required depending on timing (i.e. QM-ZM life-cycle dependent, seasonal sampling). Synthesis of reviewed materials suggests that detection success may be improved by sampling during (Sepulveda et al. 2019) and/or after (Peñarrubia et al. 2016; Gingera et al. 2017) the spawning season, near the bottom of deep waters (Amberg and Merkes 2016; Amberg et al. 2019; Marshall and Stepien 2019) and where eDNA/eRNA particles are likely to accumulate (Amberg and Merkes 2016; Amberg et al. 2019). Sites of accumulation may not coincide with optimal QM-ZM habitat and may, instead, reflect environmental patterns of transport and settlement (Amberg and Merkes 2016; Amberg et al. 2019; Shogren et al. 2019).

#### Field portable instruments (and PCR inhibition)

A primary goal in QM-ZM surveillance is early detection and rapid response. Yet, most eDNA surveys have relied on laboratory-based workflows, instrumentation and analyses, which contributes to delays in results. The adoption of field portable and/ or rapid detection devices will likely improve the ability to implement on-site QM-ZM surveillance, thereby decreasing time-to-results, even in remote and/or widely dispersed locations. Several field-friendly instruments currently exist to potentially remedy these issues and thus improve immediacy, yet all appear to suffer some form of inadequacy, most often observed via low sensitivity (as influenced by PCR inhibition). For example, rapid detection may be possible with the handheld Franklin portable

qPCR instrument (Biomeme, Philadelphia, PA). Here, eDNA results can be generated in < 1 h. Yet, high false negative detection rates have been observed (Sepulveda et al. 2018), with additional evidence to suggest that optimisation may be required for samples in which PCR inhibitors are present (Sepulveda et al. 2018; Thomas et al. 2019). PCR inhibition is expected to be less problematic in ddPCR (e.g. Hoshino and Inagaki 2012) and in LAMP (e.g. Koloren et al. 2011). Yet, Devlin and Youngbull (2019) hypothesised that, when using a field-portable ddPCR instrument (DNA Tracker), "entrained organic matter" may have led to a false positive QM-ZM detection (via chimerisation) in non-infested waters. When Williams et al. (2017) investigated the use of a hand-held, battery operated LAMP device (Gene-Z; Stedtfeld et al. 2014), PCR inhibition did not appear to be a specific issue for QM-ZM surveillance. In fact, LAMP has been proven useful for rapid, onsite surveillance of other AIS in highrisk pathways, even with novice users (Merkes 2020). Yet, as demonstrated elsewhere (Bühlmann et al. 2013; Waliullah et al. 2019), Williams et al. (2017) observed poor performance of LAMP in settings where eDNA concentrations were low, indicating a trade-off between inhibitor tolerance and lowered sensitivity. These studies cumulatively indicate that methods to combat PCR inhibition are needed to better meet management priorities for accurate and reliable early detection.

## Quantification accuracy

Several lab- and field-based studies report an observed correlation between known QM-ZM abundance and qPCR-based (Peñarrubia et al. 2016; De Ventura et al. 2017) or HTS-based (Klymus et al. 2017; Marshall and Stepien 2019) measurements (i.e. concentrations) of eDNA and/or eRNA (Marshall et al. 2021). Yet, the ability to precisely quantify QM-ZM eDNA and/or accurately interpret eDNA concentrations for abundance purposes has been called into question by at least two publications, including one in which controlled samples had been experimentally spiked with synthetic DNA at known concentrations (Sepulveda et al. 2020a) and another in which environmental factors were observed to complicate patterns in correlation between eDNA concentration and QM-ZM density (Shogren et al. 2019). Even amongst those QM-ZM studies that found correlation, two provided critical caveats regarding the precision and reliability of these estimates, citing potential complications arising from spawning activity and potential veliger presence (Peñarrubia et al. 2016; De Ventura et al. 2017). Moreover, such imprecision in eDNA-based quantification is not unique to QM-ZM. Similar findings have been observed across various systems and numerous taxa, with multiple explanations provided to account for observed variance in correlation and discrepancies in quantification accuracy. Explanations are typically complex and synergistic, but often include factors, such as PCR inhibition (e.g. McKee et al. 2015; Sigsgaard et al. 2015), choice and biases in different sampling and/or processing methodologies (e.g. Lacoursière-Roussel et al. 2016a; Hinlo et al. 2017), variability in eDNA deposition and degradation (e.g. Jo et al. 2020) and environmental and/or ecological effects (e.g. Barnes et al. 2014; Strickler et al. 2015). A recent meta-analysis

revealed that correlations between quantification measures (i.e. eDNA concentrations and known abundance) were substantially and significantly stronger in experimental lab and/or artificial pond environments than in natural systems (Yates et al. 2019). Together these observations suggest that eDNA-based abundance estimates are prone to error and require additional investigation followed by subsequent optimisation. Until improvements are made, eDNA data can currently only provide – with some reliability – rough, semi-quantitative assessments of QM-ZM abundance (e.g. on a scale from very rare to extremely common).

Although no easy and straightforward solutions exist to immediately resolve these challenges in quantification, we see two paths forward. First, investigators should compare the performance of gPCR-based estimates to ddPCR-based estimates (as in, for example, Nathan et al. 2014; Doi et al. 2015) using high-performing QM-ZM assays (e.g. see results in Sepulveda et al. 2020a; Thalinger et al. 2020). When compared to qPCR, ddPCR should provide more precise, more consistent and more reproducible quantification of eDNA (even in the presence of inhibitors) as it utilises direct, absolute measurements not reliant on potentially fallible standard curves (although, note: similar to sampling replication, technical replication improves qPCR quantification; Mauvisseau et al. 2019). Yet, while ddPCR may present a technical solution for improved quantification accuracy, it still cannot remedy the inconsistencies and/or variability observed in the actual relationship between eDNA concentration and QM-ZM abundance, which often results from ecological and environmental factors. Thus, it will be necessary to not only utilise more precise instrumentation (and/or technical approaches), but also continue to investigate QM-ZM eDNA dynamics, especially dynamics in natural settings. This combination of refinements could lead to increased quantification precision and interpretation, possibly resulting in the ability to better assess eDNA survey data, both in terms of QM-ZM infestation levels and in terms of management successes (e.g. eradication efforts, where successful removal should equate to decreases in eDNA concentration). For such advancements to be effective, surveyors must take into consideration the fate of eDNA, including rates of shed, decay and degradation.

#### Degradation in natural settings

Degradation findings from laboratory-based aquaria experiments suggest that, amongst the markers studied to date, H2B mRNA provides the best eRNA marker for finer spatiotemporal QM-ZM assessments, narrowing the window of detection to < 24 h (Marshall et al. 2021). The same experiment provided evidence that the eDNA:eRNA ratio is a helpful predictor of time since deposition, demonstrating the advantage of using both eDNA and eRNA simultaneously (Marshall et al. 2021). Quantification accuracies aside (see discussion above), future studies should evaluate these findings in outdoor mesocosm settings, as even Marshall et al. (2021) suggests lab-based experiments may oversimplify the fate of eDNA and eRNA in more natural environmental conditions. De Ventura et al. (2017) called for these types of experiments and findings from Shogren et al. (2019) indicate that environmental conditions influence
the fate (i.e. dispersion, retention and degradation) of molecular targets. We echo the recommendations of two recent publications (one review, Harrison et al. 2019 and one meta-analysis, Yates et al. 2019) and suggest that QM-ZM degradation experiments must be conducted in more natural environments before knowledge can be applied to real-world, field surveys. We also suggest that veliger presence must be controlled in these outdoor experiments; DNA within whole, microscopic organisms does not behave (i.e. degrade) the same as eDNA shed from adult mussels and may confound decay rate observations. A better understanding of the ecology of eDNA (including physical attributes and abiotic/ biotic interactions and fate; Barnes and Turner 2016) in real field settings and for improved quantification will benefit QM-ZM surveillance efforts tremendously by helping to reduce error and uncertainty in the interpretation of eDNA and/or eRNA data.

## Translating eDNA survey results into AIS management action

Dreissenids pose severe risks to invaded waters and exhibit an exceptional ability to colonise new locations. Thus, proactive eDNA surveillance has been recommended to combat the spread of QM-ZM, in the hope that early detection and rapid response will prohibit colonisation (United States Department of Interior 2017). For this to be effective, AIS managers must be able to trust the outcomes of eDNA surveys and must be able to translate eDNA results into actionable responses. This represents an area of contention. Despite the efforts of eDNA experts to improve confidence in eDNA results via developments in QA-QC measures (Goldberg et al. 2016) and careful design of eDNA assays (Klymus et al. 2020a), managers may still be reluctant to adopt eDNA approaches for surveillance and management purposes. Much of the reluctance can be attributed to perceived uncertainty in eDNA detection capabilities and, specifically, a fear of "false-positive" detections unsupported by visual survey methods (Jerde et al. 2021).

Darling et al. (2021) suggests this perceived uncertainty is due to unrealistic expectations, a disregard for the low-sensitivity often exhibited by conventional survey methods (which potentially makes them inadequate for confirming the results of extremely sensitive eDNA approaches) and poorly defined eDNA terminology. In fact, Sepulveda et al. (2020c) demonstrates that eDNA methods are mature and scientifically-defensible, with well-established protocols for preventing, detecting and quantifying detection errors (e.g. false positives, contamination). While clearly susceptible to imperfect detection (as is the case with all AIS surveillance methods), Sepulveda et al. (2020c) suggests the problem with eDNA is not the validity of the method. Instead, what prevents adoption of eDNA into AIS policy and decision-making processes is a lack of pre-defined frameworks for integration, which incorporates risks and uncertainties. Together, Darling et al. (2021) and Sepulveda et al. (2020c) suggest that greater collaboration is needed between eDNA practitioners and resource managers. There is a need to involve eDNA surveillance end-users (e.g. natural resource managers, AIS decision-makers, other stakeholders) in eDNA study design, in which these end-users provide input to jointly formulate a decision-support framework. According

to the guidance of Sepulveda et al. (2020c) and Darling et al. (2021), these frameworks should outline – in advance of eDNA sampling – criteria for discerning positive eDNA samples and subsequent action, expectations for critical QA-QC, jointly defined terminology (especially, false positives) and communication plans. These criteria should take into consideration the limitations inherent with eDNA surveillance (and conventional methods), as well as levels of confidence and/or risk acceptable by both parties. For an example of how to achieve this, see Sepulveda et al. (2020c).

Practitioners can also increase confidence by following minimum reporting guidelines. This means reporting the occurrence and subsequent handling of contamination issues (Sepulveda et al. 2020b). It also means reporting observed limits of detection and quantification (Bustin et al. 2009; Goldberg et al. 2016; Klymus et al. 2020a, b). These data allow for critical evaluations of the quality and integrity of eDNA methods, results and interpretations. Managers, interested in eDNA monitoring, should employ qualified, knowledgeable eDNA labs. Evidence suggests eDNA results are impacted by lab expertise and are most reliable (repeatable and reproducible) when practitioners are familiar with appropriate eDNA methodology and utilise high-performing assays (Hosler et al. 2017; Sepulveda et al. 2020a). Jerde (2021) outlines six essential criteria for evaluating AIS eDNA surveillance outcomes, which managers may find useful for assessing the strength of eDNA detections and for taking management action.

## Conclusions

Adherence to the optimised guidance outlined above will serve to improve and standardise molecular-based QM-ZM surveillance efforts across studies. Yet, until specific challenges are overcome, inferences beyond simple presence/absence will remain limited. As such, efforts to address critical remaining gaps are essential for advancements in the interpretation of molecular-based survey data. With continued investigation and experimentation, we may be able to further refine the levels and kinds of inference possible and, hopefully, through enhanced knowledge and improved sensitivity and reliability, provide increasingly useful information to better meet management objectives. Given the negative impacts resulting from QM-ZM invasions and the relative ease with which the species can be spread, it is likely that both species will continue to be at the forefront of developments in this field.

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